on a Bruker AM-360 apparatus using $CDCl_3$ as the solvent. A comparison of the proton NMR spectra of the starting materials and the final copolymer verified the molecular structure.

For the NMR study reported herein, the reaction compounds were PCL/PEG or PCL/F68 (see Figs. 13 and 12, respectively). However, when the polymers are used for drug delivery, further reactions may be carried out to form the triblock copolymers, PCL/PEG/PCL or PCL/F68/PCL.

Figs. 8-11 show the spectra of the starting materials PCL-diol, Pluronic F68, PEG E4500, and Denacol EX252, respectively. The spectrum of the PCL/F68 copolymer is shown in Fig. 12 and matches the proposed molecular structure shown hereinabove. Comparing the chemical shifts in the starting materials, PCL, F68, and EX252, to the shifts observed on Fig. 12, it is certain that there are PCL segments (chemical shifts at positions a, b, c, d) and F68 segments (chemical shifts at positions e and f) in the final product. A small peak at δ 0.7 ppm which has the lowest intensity should be the shift of proton h in the $-CH_3$ groups in Denacol EX252. The reaction between epoxide groups and hydroxyl end-groups was confirmed by the chemical shift at δ 3.401 ppm (proton x) which represents the protons in the linking bonds resulting from the reaction. The $-CH_2OH$ end groups in the final copolymer gave a shift at 3.415 ppm.

The spectrum of the block copolymer PCL/PEG is shown in Fig. 13. This spectrum shows the same shifts as in Fig. 12 except for proton f which represents the difference between Pluronic F68 and PEG E4500 as shown in the spectra of Figs. 9 and 10. The PCL/PEG block copolymer shown in Fig. 13 had a 75:25 molar ratio of PCL to PEG.

In the spectrum of Fig. 14, the PCL/PEG copolymer had a 60:40 molar ratio of PCL to PEG, and therefore, contained a greater proportion of PEG than the PCL/PEG copolymer shown in Fig. 13. The chemical shifts caused by the protons in Denacol EX252 which are extremely weak due to their relatively very small amounts, were deliberately enlarged. The chemical shift at 0.71 ppm (protons h) represents 6 protons in the $-CH_3$ groups in Denacol EX252 and peak r at 2.64 ppm is the shift which comes from the two protons of $-CH_2$ in the epoxide end group in Denacol EX252. After the epoxide reacted with the polymer diols, the intensity of this proton r was greatly reduced. It can be verified by the intensity ratio of h/r. Before reaction, the ratio is 3.6 as shown in Fig. 11. The ratio changed to 7.7 after the formation of the copolymer (Fig. 14). There is a trace amount of unreacted epoxide in the copolymer. This indicates that it is possible that one of the epoxide groups can be reacted with the -OH end groups of the poly-diol while leaving the other epoxide group free so that an epoxide-capped copolymer would be formed if excess Denacol EX252 is used.

Example 18:

Heparin and albumin were chemically linked with the terminal hydroxyl groups of the block copolymer through use of multi-functional epoxide compounds, illustratively Denacol EX521. In this embodiment, Denacol EX521, with five epoxide groups per molecule, was used as a linking reagent instead of the difunctional Denacol EX252 so that more free epoxide groups would be available for coupling reactions. An excess of Denacol EX521 was reacted with the terminal hydroxyl groups of the polymer particles to form epoxide-capped ends. The coupling of heparin

or albumin to the PCL-based polymer particles is the same reaction between the free epoxide group on the polymer ends and amino, hydroxyl, or other functional groups in albumin and heparin molecules, as described hereinabove in the section on epoxy-derivatization.

Triblock ABA and BAB copolymers of PCL and PEG or F68 of the type described in Example 17 were used to make nanoparticles. A specific illustrative preparation scheme is as follows: 100 mg polymer was dissolved in 5 ml methylene chloride and 1 ml acetone. This polymer solution was added, with sonication at 55 Watts of energy output, into 20 ml distilled water. Sonication was continued for a total of 10 minutes to form an oil-in-water emulsion. Organic solvent was evaporated at room temperature with stirring for 16 hours. Nanoparticles were recovered by ultracentrifugation at 145,000 g, resuspended, and lyophilized.

In a specific illustrative embodiment for the surface modification of PCL-based nanoparticles, 50 mg polymer nanoparticles were suspended in 10 ml pH 5.0 borate buffer (0.05 M). An excess of Denacol EX 521 (0.8 g) was dissolved in 5 ml of the same buffer and added into the polymer particle suspension. A catalyst, zinc tetrafluoroborate ($Zn(BF_4)_2$; 14 mg), was added with stirring. The reaction mixture was shaken at 37° C for 30 minutes. The particles were collected by centrifugation and the excess epoxide compound was removed by washing the separated particles with water. The result was epoxide-capped polymer particles.

The epoxide-capped polymer particles were resuspended in 10 ml borate buffer and 20 mg heparin or albumin was added with stirring. The reaction was permitted to continue for 5 to 10

hours at 37° C. The final product was collected by centrifugation. Free heparin or albumin was removed by washing the nanoparticles three times with water.

In order to measure the amount of heparin or albumin coupled to the polymer particles, radiolabeled heparin (^{3}H-heparin) and albumin (^{14}C-albumin) were used in the coupling reaction. About 5 mg coupled particles were dissolved in 5 ml of methylene chloride. The organic solution was washed three times with water (7 ml). The concentration of heparin or albumin in the combined aqueous extracts was measured by liquid scintillation counting and the amount of total heparin or albumin in the polymer particles was calculated from a calibration plot.

Table 14 shows the results of coupling albumin (BSA) to various block copolymer particles. The nanoparticles made of the polymer PCL/EX252/PCL is the expanded PCL-diol, compound 33 of Fig. 7.

Table 14

Specimen	Amount of BSA (mg)	BSA % (w/w) coupled to polymer	Efficiency of BSA Coupling (%)
PCL/F127/PCL	1.37	15.40	38.50
PEG/PCL/PEG	1.19	11.37	28.43
PCL/PEG/PCL	1.25	13.17	32.43
PEG/PCL/PEG/PCL/PEG	1.36	13.22	33.05
PCL/F68/PCL	0.82	6.46	16.15
PCL/EX252/PCL	0.33	3.51	8.78

Referring to Table 14, it is obvious that the amount of albumin coupled to the nanoparticles varies with the hydrophobicity of the polymer. More hydrophilic polymers result in higher coupling. Since the coupling takes place at the end of the polymer molecule, the molecular weight of the polymer would be an important factor in coupling efficiency. The higher the molecular weight, the lower the amount of albumin that can be coupled. A person of ordinary skill in the art, in the practice of the invention, would have to balance the desired molecular weight required for mechanical strength against the biomolecular coupling required for a given application.

For solid dosage forms, *e.g.*, implants, requiring long-term release, a hydrophobic polymer is useful. Hydrophilic polymers are permeable to water or tissue fluid, and will consequently, bioerode more quickly. From the standpoint of making nanoparticles, the hydrophobic/hydrophilic balance should be adjusted so that the polymer can form nanoparticles without an external emulsifier. If the polymer is too hydrophilic in nature, or too hydrophobic, an emulsifier will be required to form nanoparticles. Further, if the polymer is too hydrophilic, it will be difficult to recover. Of course, hydrophilic polymers will entrap more hydrophilic drug and hydrophobic polymers will entrap more hydrophobic drug. A person of ordinary skill in the art can easily control these properties by determining the appropriate number of hydrophobic and hydrophilic segments, as well as their relative positions (*e.g.*, BAB or ABA), in the multi-block polymers.

The stability of the albumin-coupled nanoparticles was tested in a diffusion chamber containing phosphate buffer, pH 7.4, at 37° C. Nanoparticles of PCL/F68/PCL made in accordance with Example 18 were suspended in buffer and continuously shaken. Periodically,

samples of buffer was removed and replaced with fresh buffer. Radioactivity of the removed buffer samples was measured by liquid scintillation counting. In this manner, the stability of albumin (BSA) coupled to PCL/F68/PCL copolymer was monitored over a 60 day period and compared to a polymer comprising a physical mixture, or dispersion, of BSA with the PCL/F68/PCL nanoparticles. It is to be noted, that the physical mixture of albumin with nanoparticles is not considered to be part of the invention.

The results are shown in Fig. 15 which is a graphic representation of the percent of albumin remaining in the PCL/F68/PCL nanoparticles as function of time in days. Referring to Fig. 15, the chemically coupled albumin was very stable. More than 90% of the coupled albumin remained after 62 days of incubation. The physically mixed albumin/polymer specimen exhibited faster leakage than the coupled specimen during the first 5 days. The high molecular weight of albumin may impede its diffusion from the polymer particles.

Table 15 shows the results of coupling heparin to various block copolymer particles. Approximately 5% w/w heparin was coupled to particles of each identified copolymer.

Table 15

Specimen	Amount of Heparin (mg)	Heparin % (w/w) coupled to polymer	Efficiency of Heparin Coupling (%)
PEG/PCL/PEG	0.64	5.87	14.68
PCL/F68/PCL	0.51	4.95	12.38
PCL/EX252/PCL	0.46	5.05	12.63

Heparin-coupled nanoparticles were subjected to standard APTT testing. No clotting occurred over 200 seconds for dog plasma treated with heparin-coupled nanoparticles, confirming the antithrombogenic effect of the coupled heparin. In comparison, un-heparinized particles clotted within 20-30 seconds.

Figs. 16A through 16C are graphical representations of the stability of the heparin-coupled nanoparticles of Table 15 expressed as % bound heparin remaining over time in days. The chemically coupled heparin is substantially more stable than the physically mixed. About 85% of the heparin remained in the chemically coupled particles after 43 days as compared to 15% in the physically mixed samples.

Example 19:

U86 and dexamethasone were incorporated into nanoparticles comprising PCL-based copolymers. The nanoparticles were prepared by the in-solvent emulsification-evaporation technique described above (see, Example 18). However, since the block copolymers contain both hydrophobic and hydrophilic features, a surfactant is not necessarily required to form the initial oil-in-water emulsion.

The PCL-based polymer and hydrophobic drug were dissolved in an organic solvent, methylene chloride. The organic phase was sonicated in an aqueous phase, which in this particular embodiment was a sodium phosphate buffer (pH 8.0), to form an oil-in-water emulsion. The organic solvent was evaporated at room temperature with stirring. The nanoparticles were

recovered by ultracentrifugation and dried by lyophilization. The hydroxyl end groups on the block copolymers allowed heparin to couple on the particle surface.

In a specific illustrative embodiment, dexamethasone (35 mg) was dissolved in a combination of 0.5 ml acetone and 0.3 ml ethanol. The drug solution was mixed into a polymer solution (100 mg) dissolved in 5 ml methylene chloride. The organic phase, containing drug and polymer, was emulsified with sonification at 55 Watts of energy output, into 20 ml 1% PVA solution for 10 minutes over an ice bath to form an oil-in-water emulsion. The organic solvent was evaporated at room temperature for 16 hours. The nanoparticles, thus-formed, were recovered by ultracentrifugation, washed three times with water, and lyophilized.

Table 16 shows the mean particle size, drug loading, and heparin coupling to U86-containing nanoparticles. Anti-thrombogenic activity was confirmed by the APTT test which showed no clotting in greater than 200 seconds for the heparinized nanoparticles. The copolymer of F68/PCL/F68 formed the smallest particles due to the long free hydrophilic Pluronic F68 chain on both ends of the copolymer. PCL/PEG/PCL block copolymer also formed small particles.

Table 16

Specimen	U86 loading (w/w)	Heparin Coupling (%)	Particle Size (nm)
F68/PCL/F68	12.8	3.86	131.2
PCL/F68/PCL	25.2	2.67	585.8
PCL/PEG/PCL	16.1	4.16	168.5

Fig. 17 is a graphical representation of the *in vitro* release of U86 from the heparinized nanoparticles expressed as % U86 released over time in days. Over a 33 day period, about 85% of the incorporated U86 is released from PCL/PEG/PCL, 75% from F68/PCL/F68, and 50% from PCL/F68/PCL. The nanoparticles remained intact after 33 days in the *in vitro* environment. It is hypothesized that release of U86 from the particles in the first 30 days was primarily by diffusion. The remaining U86 will be released more slowly as the polymer degrades. Fig. 17 also shows that PLGA nanoparticles release a greater amount of drug than the PCL-based triblock copolymers.

Dexamethasone-containing nanoparticles were made in accordance with this Example and incorporated into ABA-type copolymers identified on Table 17 below. Since ABA-type copolymers were used in this specific illustrative embodiment, and hence the end segments were hydrophobic, a surfactant, specifically 1% aqueous PVA solution, was employed to emulsify the medium. PCL homopolymer (PCL/EX252/PCL), the expanded PCL-diol which is compound 33 on Fig. 7, was also used to make dexamethasone-containing nanoparticles for comparative purposes.

Table 17 shows the particle size, drug loading and results of standard APTT tests of heparin-coupled, dexamethasone-containing nanoparticles. The PCL/F68/PCL nanoparticles were particularly small. All particles showed good anti-thrombogenic activity.

Table 17

Specimen	Dexamethasone loading (w/w)	Particle Size (nm)	Thrombin Time (sec.)
PCL/PEG/PCL	33.9	117.5	>200 sec.
PCL/F68/PCL	22.1	72.2	>200 sec.
PCL/EX252/PCL	28.7	177.0	>200 sec.

Fig. 18 is a graphical representation of the % dexamethasone released *in vitro* over time, in days, for the nanoparticles described on Table 17. Within 21 days, about 80% of the incorporated dexamethasone was released from PCL/F68/PCL, 65% from PCL/PEG/PCL, and 50% from the PCL homopolymer. Smaller particle size and lower drug loading resulted in quicker release in the first three days as demonstrated by the PCL/F68/PCL nanoparticle. On the other hand, larger particles with higher drug loading demonstrated longer periods of sustained release as shown by the results for the PCL/PEG/PCL and PCL/PCL/PCL nanoparticles.

The block copolymers of the present invention can also be used as a matrix carrier for controlled release of biomacromolecules, such as albumin (BSA). Films containing 15% BSA were made from ABA-type block copolymers and PCL homopolymers by hot compression molding at 130° F and 1 ton of pressure. The resulting films of about 150 μm thickness were cut into 1 x 1 cm pieces and shaken in pH 7.4 phosphate buffer at 37° C. The amount of BSA released *in vitro* from the films was monitored by measuring absorbance at 595 nm using a BIO-RAD Protein Assay reagent (Bio-Rad Company, Hercules, CA). The results are shown in Fig. 19 which is a graphical

representation of the *in vitro* release of BSA expressed as the % BSA released over time in days. Referring to Fig. 19, it is obvious that the release of albumin from PCL/PEG copolymers is much higher than the release from the PCL homopolymer. This suggests that the release of high molecular weight proteins, which are typically hydrophilic, from a copolymer matrix is positively related to its hydrophobicity.

Contact angle measurements, which relates to the interfacial tension between solid polymer particles and water, were made to assess the hydrophilicity/hydrophobicity of several hydroxy-terminated triblock copolymers of the present invention, PCL/F68/PCL and PCL/PEG/PCL, as a function of molar ratio of hydrophobic to hydrophilic components. The results are shown below in Table 18. If the contact angle is small, the polymer surface is hydrophilic and *vice versa*. Hydrophilicity/hydrophobicity may be an important parameter in the cellular uptake of the formed nanoparticles in practical embodiments, such as treatment or prevention of restenosis and immunization with orally administered vaccines. In the latter case, the uptake of hydrophobic particles, such as polystyrene particles, by the Peyer's patches is greater than the uptake of more hydrophilic particles, such as PLGA particles.

Table 18

CONTACT DATA		
PCL/F68/PCL		
	Molar Ratio of F68 (%)	Contact Angle ± Standard Deviation F68
1	0.000	60.220 ± 0.280
2	10.000	49.730 ± 1.520
3	33.000	34.470 ± 1.360
4	40.000	24.330 ± 1.380
5	50.000	20.460 ± 1.470
6	58.000	16.140 ± 1.020
PCL/PEG/PCL		
	Molar Ratio of PEG (%)	Contact Angle ± Standard Deviation PEG
1	0.000	60.220 ± 0.280
2	30.000	39.200 ± 1.110
3	50.000	30.020 ± 1.900
4	58.000	18.550 ± 1.320
5	80.000	10.780 ± 1.900

The foregoing demonstrates that the PCL block copolymers of the present invention can be formed into nanoparticles, heparin can be covalently bound to the surface to confer anti-coagulant activity to the nanoparticles, and proteins and/or peptides can be bound to the surface and released therefrom. Of course, the PCL-based copolymers of the present invention are

derivatizable, and can thus, be reacted with a variety of bioactive agents or surface modifiers. In some embodiments, no detergents are necessary for the formation of nanoparticles. Furthermore, the unique formulations permits a far wider range of breakdown duration times than possible with standard PCL. Breakdown times can range from less than an hour to months, and even as much as three years based on reports. See, for example, Darney, *et al.*, Fertility and Sterility, Vol. 58, pp. 137-143 (1992); Darney, *et al.*, Am. J. Obstet. Gynecol., Vol. 160, pp. 1292-1295 (1989); and Ory, *et al.*, Am. J. Obstet. Gynecol., Vol. 145, pp. 600-604 (1983).

In addition to nanoparticles, it should be noted that the novel PCL-based copolymers of the present invention, and methods of making same, are applicable to the manufacture of microparticles, nanoparticles, coatings, and biodegradable monolithic drug depots or polymer matrices and/or devices, such as surgical sutures, catheter tips, urinary catheters, *etc.*

III. Method of Use Embodiments:

The nanoparticle form is particularly suited for catheter-based local drug delivery at any site which can be accessed through the vasculature, or by other interventional means. Therefore, the nanoparticles of the present invention are contemplated for use in catheter-based delivery systems, particularly in interventional cardiology applications and systems and in the treatment of the vasculature. Active agents for these applications, include, without limitation, dexamethasone, corticosteroids, thrombolytic drugs, calcium channel blockers, anti-platelet action drugs, anti-proliferative agents, such as U86, cytoskeletal inhibitors, DNA, anti-inflammatories, and immunosuppressants.

(1) Prevention of Restenosis

In a specific method of use aspect of the invention, the nanoparticles are useful for local intravascular administration of smooth muscle inhibitors and antithrombogenic agents as part of an interventional cardiac or vascular catheterization procedure, such as a balloon angioplasty. Due to their small size, the nanoparticles may penetrate the arterial wall, for example, and freely enter extracellular spaces.

Nanoparticles are made particularly suitable for intravascular use by co-incorporation of one or more additives to reduce thrombogenicity and enhance extracellular matrix adhesion. The additives specifically contemplated for this purpose include detergents or surfactants such as polyvinyl alcohol, heparin, albumin, cytokines, and various lipids including phospholipids and fatty acids, or a combination thereof. Surface modification with the detergent, DMAB,

produced the best results, in terms of retention, as shown in the experimental results reported hereinabove (see Tables 6 and 7). Modifying the surface charge of the nanoparticles, imparting mucoadhesive properties to the nanoparticles, and loading the nanoparticles with albumin further increased efficacy.

Model bioactive agents for this embodiment of the invention include the hydrophobic drugs, U86 and dexamethasone. However, in a specific advantageous embodiment, cytochalasin B was formulated into PLGA nanoparticles in accordance with Example 20 hereinbelow.

For treatment of restenosis of vascular smooth muscle cells, preferred therapeutic agents include protein kinase inhibitors, such as staurosporin or the like, smooth muscle migration and/or contraction inhibitors such as the cytochalasins, suramin, and nitric oxide-releasing compounds, such as nitroglycerin, or analogs or functional equivalents thereof. Cytochalasins are believed to inhibit both migration and contraction of vascular smooth muscle cells by interacting with actin. Specifically, the cytochalasins inhibit the polymerization of monomeric G-actin to polymeric F-actin, which, in turn, inhibits the migration and contraction of vascular smooth muscle cells by inhibiting cell functions requiring cytoplasmic microfilaments. The cytochalasins include mold metabolites exhibiting an inhibitory effect on target cellular metabolism, including prevention of contraction or migration of vascular smooth muscle cells. Cytochalasins are typically derived from phenylalanine, tryptophan, or leucine and are described more particularly in International application WO 94/16707 published on August 4, 1994; WO 94/07529 published on April 14, 1994; and Japanese Patent Nos 72 01,925; 72 14,219; 72

08,533; 72 23,394; 72 01924; and 72 04, 164. The text of the cited publications is incorporated and included herein by reference. Exemplary molecules include cytochalasin A-H and J-S: chaetoglobosin A-G, J, and K; deoxaphomin, proxiphomin, protophomin, zygosporin D-G, aspochalasin B-D and the like, as well as functional equivalents and derivatives. Cytochalasin B is used in this example as a model, and preferred, compound.

While the present example directly applies cytochalasin-bound nanoparticles to vascular tissue, it is to be understood that the invention clearly contemplates the surface modification of the nanoparticles so as to include binding proteins/peptides, such as vascular smooth muscle cell binding proteins, to target the nanoparticles. Vascular smooth muscle binding proteins include antibodies (*e.g.*, monoclonal and polyclonal affinity-purified antibodies, $F(ab')_2$, Fab', Fab, and Fv fragments and/or complementary determining regions (CDR) of antibodies or functional equivalents thereof; growth factors, cytokines, and polypeptide hormones and the like; and macromolecules recognizing extracellular matrix receptors, such as integrin and fibronectin receptors. In addition, binding peptides for targeting the nanoparticles would include binding peptides for intercellular stroma and matrix located between and among vascular smooth muscle cells. These peptides are associated with epitopes on collagen, extracellular glycoproteins, such as tenascin, reticulum and elastic fibers and other intercellular matrix materials.

Example 20:

150 mg PLGA was dissolved in 5 ml methylene chloride and 15 mg cytochalasin B (Sigma Chemical Co., St. Louis, MO) was dispersed in the polymer solution. Acetone (about 4

ml) was added drop-wise, with stirring, until a clear solution, or organic phase, was formed. The organic phase was emulsified in 20 ml 2.5% PVA solution with sonication to form an oil-in-water emulsion. The oil-in-water emulsion was stirred for 16 hours on a magnetic stir plate to evaporate the organic solvents. The resulting nanoparticles were recovered by ultracentrifugation, washed until free from un-entrapped cytochalasin B and lyophilized for 48 hours. A typical yield for this procedure is about 60%. The nanoparticles have about 7.08% w/w drug loading and an average particle size of 145.4 $\pm$ 44.1 nm.

In order to evaluate cellular uptake of cytochalasin B-loaded nanoparticles, a fluorescent dye, coumarin-6, was incorporated into the nanoparticle formulation of Example 20 Specifically, approximately 0.1% by weight coumarin-6 was dissolved into the organic phase prior to emulsification. The uptake of cytochalasin-B and subsequent retention by B054 primate smooth muscle cells (passage #25) in tissue culture. The target cells were plated out in 100 mm plates for 24 hours prior to use at 2.5 x 10^5 cells/plate (a confluent monolayer for the culture cell). The target cells were exposed to 5 ml/plate cytochalasin B-containing nanoparticles made in accordance with this example (10 μg/ml in complete media) for one hour at 37° C. Then, the monolayer was washed two times with 10 ml complete media, and re-supplied with 10 ml complete media.

The cells were harvested by trypsin/EDTA cell removal, with low speed centrifugation. The cell pellet was resuspended in PBS/2% new born calf serum/0.05% sodium azide. The uptake of nanoparticles into the cells was quantified by two methods: by direct measurement of

fluorescence by flow cytometry and by fluorescent spectrophotometric measurement of the extract of coumarin-6 from the cells with ethyl acetate. The results are given below in Table 19. Time "0" was harvest time, measurements were made after 2 hours and 24 hours of incubation at 37° C. The fluorescence data was collected in log scale and converted to linear via control samples. The linear values are reported FE value (fluors intensity).

Table 19

Cellular Fluorescence (flow cytometry)			Coumarin in Extracts	
Time Posted (hrs)	Fluorescencee FE	% Retention	Coumarin-6 (ng)	% Retention
0	871	100	1.63	100
2	255	29	0.56	34
24	145	16	0.29	18

Release of cytochalasin-B was evaluated *in vitro* over a 30 day period in a double diffusion chamber in accordance with the technique described herein, *i.e.*, 5 mg nanoparticles per ml physiological phosphate buffer (pH 7.4, 0.154 mM) at 37° C. The result are shown in Fig. 20 which is a graphic representation of the *in vitro* release of cytochalasin-B over time (in days) expressed as the percent of total cytochalasin-B released into the buffer from nanoparticles of the type made in Example 20. A sample of nanoparticles containing the fluorescent dye Coumarin-6 was also tested *in vitro* to ascertain whether the dye affected release of the active agent from the nanoparticles.

The therapeutically effective amount of nanoparticles will depend on several factors, including the binding affinity of any vascular smooth muscle binding protein associated with the nanoparticles, the atmospheric pressure applied during infusion, the time over which the therapeutic agent is applied and resides at the vascular site, the nature of the therapeutic agent employed, the rate of release of the therapeutic agent from the nanoparticles, the nature of the vascular trauma and therapy desired, and the intercellular and/or intracellular localization of the nanoparticles. For intravascular administration, the nanoparticles are suspended in a suspending medium suitable for injection, preferably in a concentration of 0.1 mg/ml or less to 300 mg/ml, and preferably in the range of 5 to 30 mg/ml. This concentration of nanoparticles is in excess of the therapeutically required amount and is still "fluid" for injection. For cytochalasin, a 10^{-3} M to 10^{-12} M concentration at the site of administration in a blood vessel is preferred.

In a preferred embodiment of the invention, the nanoparticles formed by the methods described hereinabove can be regionally and selectively injected into a target zone with a custom angioplasty catheter developed for this purpose since blood flow must be interrupted during the injection process. Several custom catheters which would be suitable for the purpose are currently in the investigational stage. These are the Wolinsky catheter (C.R. Bard, Inc., Billerica, MA), the Dispatch catheter (Sci-Med, Minneapolis, MN), and the Cordis Arterial Infusion catheter (Cordis Corporation, Miami Lakes, FL). US Patent No. 4,824,436 describes a catheter which has the ability to form a blood-free chamber within the artery into which fluid, such as a solution of heparin, can be delivered under pressure. US Patent No. 5,049,132

describes yet another catheter adapted for delivery of a liquid therapeutic agent. Of course, conventional catheters can be otherwise modified by a person of ordinary skill in the art to discharge the novel drug delivery system to an arterial (or other organ) wall. Further, infusion needles, or any other means of injecting nanoparticles are specifically within the contemplation of the invention.

In a method of use, the nanoparticles are injected under pressure, illustratively 2 to 10 atm, with 3-6 being preferred, to the wall of the vessel preceding, during, or subsequent to the damaging intervention, such as angioplasty. In a preferred embodiment, the nanoparticles include heparin which confers antithrombogenic properties in addition to inhibiting smooth muscle cell proliferation. In addition, surface modification with the detergent DMAB produces excellent results with respect to retention at the site of administration. The nanoparticles adhere to the intramural tissue and slowly degrade to release therapeutic agent which may be smooth muscle inhibitors, including agents that modulate intracellular Ca^{+2} and Ca^{+2} binding proteins, receptor blockers for contractile agonists, inhibitors of the sodium/hydrogen antiporter, protease inhibitors, nitrovasodilators, phosphodiesterase inhibitors, phenothiazines, growth factor receptor antagonists, anti-mitotic agents, immunosuppressive agents, antisense oligonucleotides, and protein kinase inhibitors.

In an advantageous method aspect, inducing an osmotic shock to the vessel wall with a hypertonic solution prior to, or contemporaneously with, nanoparticle administration further enhances drug entry and extracellular matrix penetration.

Although disclosed in terms of prevention of restenosis following angioplasty, the method of the present invention can be applied to any balloon catheter procedure for such conditions as coronary artery disease, benign prostatic hypertrophy, malignant disorders of various tissues available to tubular access, occlusions in peripheral or cardiac vasculature, clearing and restoring prostatic and other intrusions in the urethra, opening fallopian tubes, and dilating esophageal strictures. Tissue injury and resulting proliferation of smooth muscle cells is often a contributing factor to complications from these procedures. Thus, the treatment of conditions wherein the target tissue or cell population is accessible by local administration, such as by catheter, infusion needle, surgical intervention, or the like, is within the contemplation of the invention.

Specifically included is the treatment of cancer with anticancer agents incorporated into nanoparticles made in accordance with the present invention. Of course, the anti-cancer-laden nanoparticles can be surface modified to target and/or enhance retention at the site. Anti-cancer agents include, but are not limited to, alkylating agents, such as mechlorethamine, cyclophosphamide, ifosfamide, mephalan, chlorambucil, hexamethylmelamine, thiotepa, busulfan, carmustine, lomustin, lomustine, semustine, steptozocin, dacarbazine; antimetabolites, such as methotrexate, fluorouracil, floxuridine, cytarabine, mercaptopurine, thioguanine, pentostatin; natural products, such as alkaloids (*e.g.*, vinblastine or vincristine), toxins (*e.g.*, etoposide or teniposide), antibiotics (*e.g.*, such as dactinomycin, daunorubicin, bleomycin, plicamycin, mitomycin), and enzymes, (*e.g.*, L-asparaginase); biological response modifiers,

such as Interferon-α; hormones and antagonists, such as adrenocortocoids (*e.g.*, dexamethasone), progestins, estrogens, anti-estrogens, androgens, gonadotropin releasing hormone analogs; miscellaneous agents, such as cisplastin, mitoxantrone, hydroxyurea, procarbazine or adrenocortical suppressants (*e.g.*, mitotane or aminoglutethimide).

(2) Sustained Release of Protein/Peptide Vaccine for Immunization

In this embodiment, the nanoparticles can be orally administered in an enteric capsule to be delivered to the gastrointestinal tract which will result in result in uptake by the intestinal mucosa and the Peyer's patch. This embodiment is useful for immunization with protein/peptide based vaccines, but can be adapted to deliver gene therapy to the Peyer's Patch lymphoid tissue.

Conventional methods of immunization generally require multiple injections at certain time intervals to achieve the desired protective immune response. Thus, multiple contacts with health care personnel are necessary. This is associated with a high "drop out" rate and a lack of cost-effectiveness, particularly in developing countries. It would be advantageous to provide an orally administered single dose vaccine immunization system which contains both an adequate priming dose as well as staged booster dose(s). In addition to securing compliance with the dosing schedule, such a dosage form would be less costly, and hence, more competitive. Cost would further be reduced for oral dosage forms which do not require needles, syringes, *etc.*

The sustained release of antigens from nanoparticles, and its subsequent processing by the macrophage and presentation to the immune system, results in an immune response. A single dose oral vaccine using capsule-protected nanoparticles of the type made in accordance with the present invention has been shown to achieve an immune response comparable to that of the conventional method of subcutaneous immunization with alum tetanus-toxoid. The capsule is designed to protect the nanoparticles and encapsulated antigen from gastric enzymes and acidic pH, and to release the enclosed antigen loaded nanoparticles in a burst in the ileum for optimal uptake by the gut-associated lymphoid tissue (and subsequent delivery to the mesenteric lymph nodes) in order to induce an immune response.

The capsule may comprise a protective time-release capsule of the type known in the prior art, and preferably is an osmotically controlled, time-release capsule of the type disclosed in USPN 5,229,895 issued on July 20, 1993, the disclosure of which is incorporated herein by reference. However, any capsule coated with enteric polymers can be used for the purpose. Such enteric polymers include cellulose acetate phthalate, shellac, Eudragit (sold by Rohm Pharmaceutical, Philadelphia, PA), *etc.* that bypass the acidic pH of the stomach and dissolve in the intestine. The time of release of the capsule contents depends upon the number of polymer coats and structure as is known in the art.

In the particular embodiment described herein, the nanoparticles are contained in a PORT™ system capsule (TSRL, Ann Arbor, MI) which is an oral drug delivery system designed to bypass the stomach and release a dose of drug to the gastrointestinal tract at specific times.

The design of the PORT™ system is based on controlling the flux of water into a gelatin capsule via a polymer film coating which regulates water flux into the capsule. As the capsule travels down the gastrointestinal tract, pressure builds inside the capsule from the influx of water and forces the contents out in a pulse. The influx of water is regulated by varying the thickness of the polymer film coating on a gelatin capsule wall. The coatings used in this particular embodiment were cellulose acetate which regulates water flux into the capsule and cellulose acetate phthalate which resists stomach acid, but dissolves at intestinal pH. As the amount of coating applied to capsule is increased, the permeability and water flux decreases. The decrease in water flux decreases the rate of pressure build-up within the capsule, thereby prolonging the time of the pulse. The pulse times can illustratively range from 4 to 9 hours for film coatings of 4 to 11%.

In addition to containment in a controlled-release capsule, the nanoparticles can be adapted to have staged, variable breakdown periods to achieve priming and booster doses. Formulation of a biodegradable polymeric non-antigen-containing sealing coat(s) which delays hydrolysis of the biodegradable polymer, and surface modification as described herein, are several of the techniques which may be used to vary the breakdown rate.

Although the following example is directed to the use of nanoparticles for the delivery of tetanus-toxoid vaccine as the model protein-based vaccine, it is to be understood that the system may be useful for delivery of other vaccines, or combinations of vaccines, to achieve long-term protective immune responses against any vaccine-preventable disease. Illustrative examples are

bacterial vaccines such as tetanus, cholera toxin, hepatitis B, acellular pertussis, Staphylococcus enterotoxin B, pneumococcus, Staphylococcus and Streptococcus antigens, and others, including combined diphtheria, pertussis, and tetanus (DPT); E. Coli (enteropathogenic); and viral vaccine proteins, such as all AIDS antigens, viral proteins (*e.g.*, influenza virus proteins, adenovirus, and others); live virus in microcapsules (*e.g.*, attenuated poliovirus), Hepatitis viral components, Rotavirus components.

Orally administered controlled release nanoparticles can induce a secretory immune response (IgA) in addition to a systemic immune response (IgG). This would be particularly useful for the prevention of respiratory, vaginal, and gut-associated mucosal infectious diseases.

Example 21:

Tetanus-toxoid (provided by the Serum Institute, Pune, India) was loaded into PLGA in the water-in-oil-in-water emulsification technique of Example 10 hereinabove. The technique produced a 57% entrapment efficiency with 12% antigen loading. Particle size distribution studies revealed a uniform particle distribution with a mean particle diameter of 154.3 ∓ 82.7 nm. The *in vitro* release rate of tetanus toxoid from the PLGA nanoparticles into a phosphate buffered saline at 37° C approximates first order kinetics.

More particularly, tetanus toxoid and a viscosity enhancer, Pluronic F-125 (BASF, Parsippany, NJ), are dissolved in water. PLGA (50:50, molecular weight 90,000, inherent viscosity, 1.07; Birmingham Polymers, Inc, Birmingham, Alabama) is dissolved in methylene chloride (3% w/v). The tetanus toxoid solution and the PLGA solutions are sonicated to form a

water-in-oil primary emulsion. The primary emulsion is then emulsified into an aqueous solution of PVA (2.5% w/v) to form a water-in-oil-in-water emulsion. The organic solvent is then evaporated, the nanoparticles are recovered by ultracentrifugation, washed three times with water, resuspended in water and lyophilized.

Example 22:

Nanoparticles incorporating BSA and 0.05% Rhodamine dye were administered to a group of rats (male, Sprague-Dawley, 230-250 mg) in order to detect their presence in the intestinal mucosa and Peyer's patch lymphoid tissue. The nanoparticles used in this study had a particle size of 150 ∓ 48.5 nm. Fluorescent microscopy revealed significant uptake of the nanoparticles in the Peyer's Patch lymphoid tissue.

Example 23:

The use of the nanoparticles of the present invention as a drug delivery device for vaccines has been demonstrated by studies in rats. Tetanus Toxoid loaded nanoparticles (15 *Lf*) were prepared and subcutaneously injected in rats. The immune response, as measured by IgG, μg/ml, was compared to the immune response in rats to which conventional Alum-Tetanus Toxoid conjugate (Pasteur-Merieux through US supplier, Connaught Laboratories, Inc., Swiftwater, PA; 5 *Lf*) had been subcutaneously administered. The results are shown on Fig. 21 which is a graphic representation of the immune response, as measure by IgG, μg/ml, at 21 days post-immunization and 30 days post-immunization.

The immune response in the short-term was virtually identical. The nanoparticles continue to release Tetanus Toxoid for 30 days, thus prolonging the sensitization-exposure period and enhancing the long term immune response. Further, the results demonstrated that the immunogenicity of Tetanus Toxoid is not adversely affected by the nanoparticle formulation procedures.

As demonstrated above, the nanoparticles of the present invention can be adapted for oral administration, as well as intravascular or subcutaneous administration, for sustained release of drugs or vaccines or used as an immunological adjuvant for immunization. Vaccines, as well as gene therapy for the paraintestinal lymphoid system, can be orally administered.

In addition to the foregoing, nanoparticles suitable for vaccination can be administered via the following routes: intramuscular, subcutaneous, oral, nasal, intraperitoneal, rectal, and vaginal.

(3) Gene Therapy

The nanoparticles can be used to deliver genetic material in a targeted manner. In this application, the nanoparticles can be formulated for administration via the oral route or the mucous membrane. The nanoparticles are capable of sustained administration of gene therapy, particularly to the lymphoid system surrounding the ileum as described hereinabove.

However, nanoparticles containing genetic material can also be devised and targeted for site-specific delivery to other cells or tissue types by injection and/or implantation. Also specifically contemplated are genetic material suitable for the DNA or anti-sense treatment of

cardiovascular disease, including platelet-derived growth factor, transforming growth factors, alpha and beta, fibroblast growth factors (acidic and basic), angiotensin II, heparin-binding epidermal growth factor-like molecules, Interleukin-1, alpha and beta, Interleukin-6, insulin-like growth factors, oncogenes (c-myb, c-myo, fos, and others), proliferating cell nuclear antigen, cell adhesion molecules (intracellular adhesion molecules, vascular cell adhesion molecules, and others), and platelet surface antigens (IIb /IIIa and others).

In another illustrative embodiment, the nanoparticles of the present invention may be used as a carrier for nucleic acids, such as an osteotropic gene or gene segment. The nanoparticles have the capability of transferring nucleic acids into bone cells and tissues for promoting bone growth and regeneration. In one specific embodiment, an osteotropic gene or gene segment is transferred into bone progenitor cells to stimulate progenitor cells and promote increased bone formation. The DNA-carrying nanoparticles may be injected to the site, which may be bone or skeletal connective tissues, such as tendons, cartilage, and ligaments. Specific examples include bone morphogenic proteins (BMP2 and 4 and others), transforming growth factor, such as TGF-β1-3, activin, phosphoproteins, osteonectin, osteopontin, bone sialoprotein, osteocalcin and other vitamin-k dependent proteins, glycoproteins, such as aggrecan, glycan, and others, and collagen (I, II, and others). Further specific examples are described in co-pending US patent application numbers 08/199,780 filed on February 18, 1994 and 08/316,650 filed on September 30, 1994, assigned to the assignee hereof, the disclosures of which are incorporated by reference herein.

Regulatory factors involved in bone repair are known to include systemic hormones, cytokines, growth factors, and other molecules that regulate growth and differentiation. Various osteoinductive agents have been purified and shown to be polypeptide growth-factor-like molecules. These stimulatory factors are referred to as bone morphogenetic or morphogenic proteins (BMPs), and have also been termed osteogenic bone inductive proteins or osteogenic proteins (OPs). Several BMP genes have now been cloned and the common designations are BMP-1 through BMP-8.

BMPs 2-8 are generally thought to be osteogenic, although BMP-1 is a more generalized morphogen (Shimell, *et al.*, 1991). BMP-3 is also called osteogenin (Luyten, *et al.*, 1989) and BMP-7 is also called OP-1 (Ozkaynak, *et al.*, 1990). BMPs are related to, or part of, the transforming growth factor-β (TGF-β) superfamily, and both TGF-β1 and TGF-β2 also regulates osteoblast function (Seitz, *et al.*, 1992. Several BMP (or OP) nucleotide sequences and polypeptides have been described in US Patents, *e.g.*, 4,795,804; 4,877,864; 4,968,590; 5,108,753; including specifically BMP-1 disclosed in 5,108,922; BMP-2A in 5,166,058 and 5,103,649; BMP-2B in 5,013, 649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6 in 5,187,076; BMP-7 in 5,108,753, and 5,141,905; and OP-1, COP-5 and COP-7 in 5,011,691. In addition, an article by Wozny, *et al.* is incorporated herein by reference to describe BMP molecular clones and their activities. The cited literature, including the patent literature specifically, also teaches how to prepare an osteotropic gene segment or cDNA.

Other growth factors or hormones that have been reported to have capacity to stimulate new bone formation include acidic fibroblast growth factor, estrogen, macrophage colony stimulating factor, and calcium regulatory agents such as parathyroid hormone. The use of bone stimulating proteins and polypeptides, particularly recombinant BMPs, has also been investigated.

In the instant invention, nucleic acid segments are transferred into bone progenitor cells or tissues at the site *in vivo*. The nucleic acid segment may be DNA (double or single-stranded) or RNA (*e.g.*, mRNA, tRNA, rRNA); it may be a "coding segment", and antisense nucleic acid molecule. Thus, the nucleic acid segments may be genomic sequences, including exons and introns alone or together, or coding cDNA regions, or in fact any construct that one desires to transfer to a bone progenitor cell and virtually any form, such as naked DNA or RNA, including linear nucleic acid molecules and plasmids, or as a functional insert within the genomes of various recombinant viruses, including viruses with DNA genomes and retroviruses.

The invention may be employed to promote expression of a desired gene in bone cells or tissues and to impart a particular desired phenotype to the cells. This expression could be increased expression of a gene that is normally expressed , or it could be used to express a gene that is not normally associated with bone progenitor cells in their natural environment. Alternatively, the invention may be used to suppress the expression of a gene that is naturally expressed in such cells and tissues, and again, to change or alter the phenotype. Gene

suppression may be a way of expressing a gene that encodes a protein that exerts a down-regulatory function, or it may utilize antisense technology.

An osteotropic gene is a gene or DNA coding region that encodes a protein, polypeptide, or peptide that is capable of promoting, or assisting in the promotion of, bone formation, or one that increase the rate of primary bone growth or healing. In addition, an osteotropic gene may be capable of stimulating the growth or regeneration of skeletal connective tissues, such as tendon, cartilage, and ligament. Bone progenitor cells refer to any or all of those cells that have the capacity to ultimately form, or contribute to the formation of, new bone tissue. They specifically include various cells in different stages of differentiation, such as stem cell, macrophages, fibroblasts, vascular cells, osteoblast, chondroblasts, osteoclasts, and the like. Osteotropic genes and the proteins that they encode include, for example, systemic hormones, such as parathyroid hormone (PTH) and estrogen; many different growth factors and cytokines; chemotactic or adhesive peptides or polypeptides; molecules such as activin (US Patent No. 5,208,219, incorporated herein by reference), specific bone morphogenetic proteins (BMPs); and growth factor receptor genes.

Examples of suitable osteotropic growth factors include the transforming growth factor (TGF) family, specifically TGFs 1-4, and particularly TGF-α, TGF-β1, TGF-β2 (US Patent Nos. 5,168,051; 4,886,747; and 4,742,033, each incorporated herein by reference); and fibroblast growth factors (FGF), such as acidic FGF and kFGF; granulocyte/macrophage colony stimulating factors (GMCSF); epidermal growth factor (EGF); platelet derived growth factor

(PDGF); insulin-like growth factors (IGF), including IGF-I and IGF-II; and leukemia inhibitory factor (LIF), also known as HILDA and DIA.

Preferred osteotropic genes and DNA segments are those of the TGF superfamily, such as TGF-α, TGF-β1, TGF-β2, and the members of the BMP family of genes. Of course, the original source of a recombinant gene or DNA segment need not be of the same species as the animal to be treated. In this regard, it is contemplated that any recombinant PTH, TGF, or BMP gene may be employed, such as those from human, mouse, and bovine sources. Gene and DNA segment refer to a DNA molecule that has been isolated free of total genomic DNA of the species from which it was obtained. Included within the term DNA segment are DNA segments and smaller fragments of such segments, and also recombinant vectors, including for example, plasmids, cosmids, phage, retroviruses, adenoviruses, and the like.

The nanoparticles of the present invention may comprise one or more osteotropic genes or nucleic acid segments, in combination, or in combination with other proteins, peptides, or pharmaceutically active agents, and/or surface modifying agents.

Example 24:

In a specific embodiment illustrating use of nanoparticles of the present invention for delivery of DNA, or DNA fragments, luciferase marker DNA was incorporated into PLGA nanoparticles in accordance with the principles of the invention.

COS cells (mouse kidney epithelium) were transfected *in vitro* using the pGL2 plasmid expression vector which encodes luciferase. A standard transfection protocol was used. In

brief, COS cells plated the day before were exposed to DNA (luciferase, Promega, Los Angeles, CA) for 2.5 hours in a standard cell culture medium, which medium lacked serum. The cells were washed and then cultured in a medium with 10% serum supplementation for 60 additional hours.

In an experiment designed to evaluate the sustained release of DNA, 20 mg of plasmid DNA was complexed with DEAE-dextran and compared to DNA enclosed in PLGA nanoparticles at a concentration of either 10 μg/ml or 20 μg/ml.

In order to make nanoparticles, PLGA (90 mg) was dissolved in 3 ml chloroform. Nuclease-free BSA (30 mg) and DNA (2 mg) were dissolved in 300 μl nuclease-free Tris - EDTA which is Tris buffer (Tris(hydroxymethyl)aminomethane; 10 mM, pH 7.4) containing 0.1 mM EDTA. The DNA-containing solution was emulsified with the PLGA polymer solution by sonication over an ice bath for 8 minutes using a microtip probe sonicator at 55 Watts of energy output. The resulting water-in-oil emulsion was further emulsified into 25 ml of 2% w/v PVA (M Wt. 30-70 K) solution in Tris-EDTA buffer saturated with chloroform using the sonicator probe at 55 Watts. The result was a water-in-oil-water emulsion. The water-in-oil-water emulsion was stirred for 18 hours with a magnetic stirrer in an open container, and then for 2 additional hours under vacuum to completely evaporate the organic solvent. Nanoparticles, thus formed, were recovered by ultracentrifugation, washed three times with Tris-EDTA buffer, and lyophilized for 48 hours. The resulting nanoparticles were stored desiccated.

Since DNA is water-soluble, it is entrapped by the emulsion procedure and distributed throughout the polymer matrix. Targeting of DNA-containing nanoparticles can be accomplished as described hereinabove with appropriate surface modifying agents, such as ferritin, antibodies which are specific to target cells, marker proteins for receptors on target cells, or the provision of a characteristic lipid coating, among others.

It should be noted specifically, that the Tris-EDTA buffer used in this specific illustrative embodiment, has antinuclease properties which prevent DNA breakdown during processing. In addition to Tris-EDTA, any other buffer or combination of buffers containing a calcium complexing or chelating agent, such as dithizone, nitrolotriacetic acid, citrates, oxalates, tartrates, and dimercaprol, is suitable for use in the practice of the invention. Calcium is a necessary cofactor in the breakdown of DNA with nucleases, therefore calcium complexing agents which competitively remove calcium ions mitigate against the loss of DNA by this mechanism. In addition to the use of calcium complexing buffers, certain proteins, such as histones, protamine or polylysine, bind nuclease and thereby block its damaging effect on the DNA. It is also advantageous to conduct the entire nanoparticle production procedure in a nuclease-free environment, such as by using nuclease-free reagents, such as nuclease-free serum albumin (available from Sigma Chemical Co., St. Louis, MO).

Luciferase activity of the nanoparticles was determined by a substrate utilization assay using a commercially available kit (Luciferase Assay System, Promega, Los Angeles, CA) substantially according to the protocol supplied by the manufacturer. In brief, cells were

homogenized in 2 ml buffer (50 mM Tris acetate, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1 mg/ml BSA). Immediately thereafter, 0.5 ml of cell culture lysis reagent (Promega) was added to the homogenate, mixed well, and incubated at room temperature for 10 minutes. To measure background activity (counts per minute, CPM), 100 μl of clarified homogenate was added to a clean microcentrifuge tube and luciferase activity was determined by scintillation counting for 1 minute at room temperature (1219 RackBeta Scintillation counter, LKB, supplied through Wallace, Inc., Gaithersburg, MD, all channels open). The same procedure was used to measure background CPM of 1 ml luciferase substrate stock solution. Once background activity was determined, homogenate and substrate were mixed and counted immediately. Enzyme activity values were normalized to 1 μl of total protein.

The results are shown graphically in Fig. 22 which is a plot of luciferase activity as CPM/μg protein for each specimen. The total amount of DNA contained in each batch of nanoparticles was considerably less than the control comparison for this experiment. Thus, the group designated PLGA-10 DNA contained 40 ng of DNA total and the group designated PLGA-20 DNA contained 80 ng of DNA. Furthermore, the sustained release of all the DNA from the nanoparticles would have actually occurred after 30 days as shown in the *in vitro* release studies conducted with the model protein, BSA. Thus, the 2.5 hours exposure to the nanoparticles constitutes a severe test of the efficacy of the nanoparticles since only minute amounts of DNA were released. Nevertheless, Fig. 22 shows significant expression above background of luciferase in three of the four DNA-containing nanoparticle groups.

In a still further illustrative embodiment of the invention, a method of making nanoparticles has been developed which does not use sonification. It has been discovered that sonication may damage genetic material. The damaging effect is magnified with larger genes. Therefore, a technique has been developed using an excess of organic solvents, such as DMSO or chloroform, and a detergent to obtain nanoparticles without the use of sonification.

Example 25:

In a specific illustrative embodiment, DNA (luciferase, 2 mg) and nuclease-free BSA (30 mg) are dissolved in 300 μl Tris-EDTA buffer to form an aqueous phase. The aqueous phase is homogenized into a PLGA polymer solution dissolved in chloroform (90 mg PLGA in 3 ml chloroform) containing 1% w/v Span-20 to form a water-in-oil emulsion. The primary emulsion is further emulsified by homogenization for 30 minutes into a 2% w/v solution of PVA in nuclease-free Tris-EDTA buffer which has been saturated with chloroform. The result is a multiple emulsion, or a water-in-oil-in-water emulsion. The organic solvent is evaporated at room temperature by stirring, uncovered, over a magnetic stirring plate for 18 hours. Then, a vacuum is applied for an additional 2 hours. The resulting nanoparticles are recovered by ultracentrifugation, washed three times with Tris-EDTA buffer and lyophilized.

Nanoparticles which include osteotropic genes and/or other materials to stimulate bone growth, may be advantageously suspending in a gelling medium which is applied to the site of need. The nanoparticles, which may be in a gelling medium, may also be intimately mixed

with another material, specifically a bone filler, such as bone cement, dental adhesives, hydroxyapatite, and bone ceramics, to hold the nanoparticles at the site of application.

Although the invention has been disclosed in terms of biodegradable polymers, in the specific embodiment directed to therapy to facilitate bone growth, nanoparticles which are, at least in part, insoluble and non-degradable are contemplated. Such nanoparticles could contain insoluble calcium phosphate crystalline mineral components, for example, to render them osteoconductive, *i.e.*, capable of facilitating new mineral formation. Such insoluble nanoparticles would be integrated into the renewed bone structure. Specifically included are all calcium phosphate mineral phases, including octacalcium phosphate, amorphous calcium phosphate, tricalcium phosphate, carbonate-apatite, and fluorapatites, as well as ceramics of all of the aforementioned. Further, calcium bisphosphonates, or other crystalline salts or free acids or mono-, bis- or polyphosphonates, would be useful as fillers and surface modifying agents. Synergistic combinations include ferric or aluminum salts of bisphosphonates.

In addition to the foregoing, it is to be understood that the nanoparticles of the present invention would find widespread application in the delivery of bioactive agents in general. The purpose of the delivery of bioactive agents may range from therapeutic to diagnostic (imaging agents), to cosmetic or nutritional. Nanoparticle-based delivery of gene therapy is expected to improve transfection of DNA over a prolonged period of time.

Although the invention has been described in terms of specific embodiments and applications, persons skilled in the art can, in light of this teaching, generate additional

embodiments without exceeding the scope or departing from the spirit of the claimed invention. Accordingly, it is to be understood that the drawing and description in this disclosure are proffered to facilitate comprehension of the invention, and should not be construed to limit the scope thereof.

What is claimed is:

Composition

1. A sustained release dosage form comprising:

nanoparticles comprising a biocompatible, biodegradable polymer core having an average diameter of less than about 300 nm, the nanoparticles having associated or incorporated therewith at least one bioactive agent and/or at least one surface modifying agent.

2. The sustained release dosage form of claim 1 wherein the average diameter of the nanoparticles is in the range of about 100-150 nm.

3. The sustained release dosage form of claim 1 wherein the average diameter of the nanoparticles is in the range of about 10-50 nm.

4. The sustained release dosage form of claim 1 wherein the biocompatible, biodegradable polymer is a synthetic polymer.

5. The sustained release dosage form of claim 4 wherein the biocompatible, biodegradable polymer is a synthetic polymer selected from the group consisting of polyesters, polyethers, polyanhydrides, polyalkylcyanoacrylates, polyacrylamides, poly(orthoesters), polyphosphazenes, polyamino acids, and biodegradable polyurethanes.

6. The sustained dosage release form of claim 5 wherein the biocompatible, biodegradable polymer is a polyester selected from the group consisting of polylactides, polyglycolides, and polylactic polyglycolic copolymers.

7. The sustained release dosage form of claim 6 wherein the biocompatible, biodegradable polymer is a polylactic polyglycolic copolymer.

8. The sustained dosage release form of claim 6 wherein the biocompatible, biodegradable polymer is a polyether selected from the group consisting of hydroxy-terminated poly (ϵ-caprolactone)-polyether or polycaprolactone.

9. The sustained release dosage form of claim 8 wherein the polyether is a polycaprolactone which is epoxy-derivatized and activated.

10. The sustained release dosage form of claim 1 wherein the biocompatible, biodegradable polymer is a naturally-derived polymer.

11. The sustained release dosage form of claim 10 wherein the biocompatible, biodegradable polymer is a naturally-derived polymer selected from the group consisting of acacia, chitosan, gelatin, dextrans, albumins, and alginates/starch.

12. The sustained release dosage form of claim 1 wherein the bioactive agent is at least one pharmaceutical agent.

13. The sustained release dosage form of claim 11 wherein the at least one pharmaceutical agent is a cardiovascular agent.

14. The sustained release dosage form of claim 13 wherein the cardiovascular agent is selected from the group consisting of stimulators, inhibitors, antithrombins, calcium channel blockers, antitensin converting enzyme (ACE) inhibitors, immunosuppressants, fish oils, growth factor antagonists, cytoskeletal inhibitors, antiinflammatory agents,

thrombolytic agents, antiproliferatives, genetic material suitable for the DNA or anti-sense treatment of cardiovascular disease, protein kinase inhibitors, smooth muscle migration and/or contraction inhibitors, and nitric oxide-releasing compounds.

15. The sustained release dosage form of claim 14 wherein the cardiovascular agent is a cytoskeletal inhibitor.

16. The sustained release dosage form of claim 15 wherein the cytoskeletal agent is cytochalasin B.

17. The sustained release dosage form of claim 12 wherein the bioactive agent is an anticancer agent.

18. The sustained release dosage form of claim 17 wherein the anticancer agent is selected from the group consisting of alkylating agents, antimetabolites, natural products (*e.g.*, alkaloids), toxins, antibiotics, enzymes, biological response modifiers, hormones, antagonists, and genetic material suitable for the treatment of cancer.

19. The sustained release dosage form of claim 12 wherein the bioactive agent is a peptide or protein-based vaccine.

20. The sustained release dosage form of claim 19 wherein the protein-based vaccine is Tetanus-Toxoid.

21. The sustained release dosage form of claim 12 wherein the bioactive agent is a nucleic acid.

22. The sustained release dosage form of claim 21 wherein the nucleic acid is DNA, RNA, or an oligonucleotide (sense or antisense) of DNA or RNA.

23. The sustained release dosage form of claim 22 wherein the nucleic acid is an osteotropic gene or gene segment, or oligonucleotide.

24. The sustained release dosage form of claim 23 wherein the osteotropic gene or gene segment is selected from the group consisting of bone morphogenic proteins (BMP2 and 4 and others), transforming growth factor, such as TGF-β1-3, activin, phosphoproteins, osteonectin, osteopontin, bone sialoprotein, osteocalcin, vitamin-k dependent proteins, glycoproteins, and collagen (at least I and II).

25. The sustained release dosage form of claim 22 further including at least one osteoconductive salt.

26. The sustained release dosage form of claim 22 wherein the nucleic acid is suitable for the DNA or anti-sense treatment of cardiovascular disease and is selected from the group consisting of platelet-derived growth factor, transforming growth factors (alpha and beta), fibroblast growth factors (acidic and basic), angiotensin II, heparin-binding epidermal growth factor-like molecules, Interleukin-1 (alpha and beta), Interleukin-6, insulin-like growth factors, oncogenes, proliferating cell nuclear antigen, cell adhesion molecules, and platelet surface antigens.

27. The sustained release dosage form of claim 22 wherein the nucleic acid is an anticancer gene selected from the group consisting of tumor suppressor genes, cytokine-

producing genes, tumor necrosis factor α-cDNA, carcinoembryonic antigen gene, lyphokine gene, toxin-mediated gene therapy, and antisense RNA of E6 and E7 genes.

28. The sustained release dosage form of claim 21 further including a protein to block nuclease activity.

29. The sustained release dosage form of claim 1 wherein the surface modifying agent is selected from the group consisting of one or more synthetic polymers, biopolymers, low molecular weight oligomers, natural products, and surfactants.

30. The sustained release dosage form of claim 31 wherein the surface modifying agent is a synthetic polymer selected from the group consisting of carboxymethyl cellulose, cellulose, cellulose acetate, cellulose phthalate, polyethylene glycol, polyvinyl alcohol, hydroxypropylmethyl cellulose phthalate, hydroxypropyl cellulose, sodium or calcium salts of carboxymethyl cellulose, noncrystalline cellulose, polaxomers, poloxamines, dextrans, DEAE-dextran, polyvinyl pyrolidone, polystyrene, and silicates.

31. The sustained release dosage form of claim 29 wherein the surface modifying agent is a natural product selected from the group consisting of proteins, peptides, sugar-containing compounds, and lipids.

32. The sustained release dosage form of claim 31 wherein the natural product is a peptide/protein selected from the group consisting of acacia, gelatin, casein, albumins, myoglobins, hemoglobins, and fibrinogens.

33. The sustained release dosage form of claim 31 wherein the natural product is a sugar-containing compound selected from the group consisting of tragacanth, sorbitol, mannitol, polysaccharides, and pectin.

34. The sustained release dosage form of claim 31 wherein the natural product is a lipid selected from the group consisting of lecithin, phospholipids, cholesterol, beeswax, wool fat, sulfonated oils, and rosin soap.

35. The sustained release dosage form of claim 29 wherein the surface modifying agent is a surfactant selected from the group consisting of non-ionic, anionic, and cationic surfactants.

36. The sustained release dosage form of claim 35 wherein the surface modifying agent is a non-ionic surfactant selected from the group consisting of polyoxyethylene sorbitan fatty acid esters, sorbitan fatty acid esters, fatty alcohols, alkyl aryl polyether sulfonates, and dioctyl ester of sodium sulfonsuccinic acid.

37. The sustained release dosage form of claim 35 wherein the surface modifying agent is an anionic surfactant selected from the group consisting of sodium dodecyl sulfate, sodium and potassium salts of fatty acids, polyoxyl stearate, polyyoxylethylene lauryl ether, sorbitan sesquioleate, triethanolamine, fatty acids, and glycerol esters of fatty acids.

38. The sustained release dosage form of claim 35 wherein the surface modifying agent is a cationic surfactant selected from the group consisting of didodecyldimethyl

ammonium bromide, cetyl trimethyl ammonium bromide, benzalkonium chloride, hexadecyl trimethyl ammonium chloride, dimethyldodecylaminopropane, N-cetyl-N-ethyl morpholinium ethosulfate.

39. The sustained release dosage form of claim 1 further including a suspending medium.

40. The sustained release dosage form of claim 39 wherein the suspending medium is selected from the group consisting of distilled water, normal saline, triglycerides, physiologic buffers, serum or serum/plasma protein constituents, and tissue culture media.

41. The sustained release dosage form of claim 39 wherein the suspending medium gels after application to the region of injection.

42. The sustained release dosage form of claim 41 wherein the suspending medium which gels is selected from the group consisting of poloxamers, Types I and II collagen or procollagen, hydrogels, cyanoacrylates, and fibrin glue.

43. The sustained release dosage form of claim 41 in intimate combination with a bone filler selected from the group consisting of bone cement, dental adhesive, hydroxyapatite, and bone ceramics.

44. The sustained release dosage form of claim 1 further including an encapsulation for the nanoparticles.

Methods of Making

hydrophobic agents

45. A method of making a sustained release drug delivery system for hydrophobic bioactive agents comprising the steps of:

(a) dissolving at least one biocompatible, biodegradable polymer in an organic solvent;

(b) dissolving the bioactive agent(s) in an organic solvent, the combined polymer and bioactive agent-containing solutions comprising an organic phase;

(c) adding the organic phase to an aqueous phase;

(d) sonicating the combined organic phase and the aqueous solution at a temperature below the melting point of the polymer and at an energy sufficient to form a stable emulsion;

(e) evaporating the organic solvent from the stable emulsion; and

(f) separating resulting nanoparticles from the remaining aqueous phase.

46. The method of claim 45 wherein the aqueous phase is an aqueous solution of an emulsifying agent.

47. The method of claim 46 wherein aqueous solution of an emulsifying agent has about 0.1% to 10% w/v emulsifying agent, and preferably about 1% to 3% w/v emulsifying agent.

48. The method of claim 45 wherein the emulsifying agent is selected from the group consisting of polyvinyl alcohol, polyoxyethylene sorbitan fatty acid esters, polyethylene

glycols, triethanolamine fatty acid esters, sodium and potassium salts of fatty acids, sodium lauryl sulphate cellulose acetate, polaxomers, and quaternary ammonium compounds.

49. The method of claim 45 comprising the further step of lyophilizing the nanoparticles.

50. The method of claim 49 wherein the lyophilizing step comprises subjecting the nanoparticles to temperatures on the order of -30° C to -55° C in a vacuum of 500 millitorr or less for at least 24-48 hours.

51. The method of claim 49 further comprising the step of sterilizing the nanoparticles.

52. The method of claim 51 wherein the step of sterilizing comprises subjecting the nanoparticles to a sterilizing radiation.

53. The method of claim 45 wherein, in the step of sonicating, the energy sufficient to form a stable emulsion is in the range of 35-65 Watts.

54. The method of claim 49 further comprising the step of modifying the surface of the resulting nanoparticles.

55. The method of claim 54 wherein the step of modifying the surface of the resulting nanoparticles comprises adsorbing at least one surface modifying agent to the nanoparticles.

56. The method of claim 55 wherein the step of adsorbing comprises the steps of suspending the nanoparticles in a solution of the at least one surface modifying agent and freeze-drying the suspension to produce a coating on the nanoparticles.

57. The method of claim 56 wherein the step of freeze-drying comprises lyophilizing the nanoparticles in a lyophilizer at -30° C to -55° C in a vacuum of 500 millitorr or less for at least 24 to 48 hours.

58. The method of claim 54 wherein the step of modifying the surface comprises epoxy-derivatization.

59. The method of claim 58 wherein epoxy-derivatization comprises the steps of partially hydrolyzing the nanoparticles to create reactive groups on the surface; and contacting the hydrolyzed nanoparticles with a reactive multifunctional epoxide to form epoxy-activated nanoparticles.

60. The method of claim 59 wherein the reactive groups are amino, anhydrides, carboxyl, hydroxyl, phenol, or sulfhydryl.

61. The method of claim 58 wherein the reactive multifunctional epoxide is selected from the group consisting of 1,2-epoxides, 1,2-propylene oxides, butane and ethane di-glycidyl ethers, erythritol anhydride, polyfunctional polyglycerol polyglycidyl ethers, and epichlorhydrin.

62. The method of claim 59 comprising the further step of reacting the epoxy-activated nanoparticles with reactive groups on one or more bioactive agents and/or surface modifying agents.

63. The method of claim 54 wherein the at least one surface modifying agent is selected from the group consisting of one or more synthetic polymers, biopolymers, low molecular weight oligomers, natural products, and surfactants.

64. The method of claim 54 wherein the step of modifying the surface comprises incorporating the at least one surface modifying agent in the polymer matrix.

65. The method of claim 64 wherein the step of incorporating the at least one surface modifying agent in the polymer matrix comprises using at least one biodegradable, biocompatible polymer in the organic phase which has a surface modifying property.

66. The method of claim 65 wherein the biodegradable, biocompatible polymer is an epoxy-derivatized and activated polycaprolactone.

67. The method of claim 65 wherein the biodegradable, biocompatible polymer is a cyanoacrylate.

hydrophilic agents

68. A method of making a sustained release drug delivery system for hydrophilic bioactive agents comprising the steps of:

(a) dissolving a biodegradable, biocompatible polymer in a nonpolar organic solvent;

(b) dissolving hydrophilic bioactive agent(s) in a semipolar organic solvent or a combination of a polar and semipolar solvent, the combined polymer and bioactive agent-containing solutions comprising an organic phase;

(c) adding the organic phase to an aqueous phase;

(d) sonicating the combined organic phase and the aqueous solution at a temperature below the melting point of the polymer and at an energy sufficient to form a stable emulsion;

(e) evaporating the organic solvent from the stable emulsion; and

(f) separating resulting nanoparticles the remaining aqueous phase.

69. The method of claim 68 wherein the aqueous phase is an aqueous solution of an emulsifying agent.

70. The method of claim 68 wherein the organic phase further includes an agent to favor partitioning of the hydrophilic bioactive agent into the organic phase upon solidification of the resulting nanoparticles.

71. The method of claim 70 wherein the agent to favor partitioning is selected from the group of covalent complexing agents, pH adjusting agents, lipids, and viscosity enhancers.

72. The method of claim 71 wherein the agent to favor partitioning is a covalent complexing agent which is a fatty acid salt.

73. The method of claim 71 wherein the agent to favor partitioning is a cationic or anionic lipid.

74. The method of claim 71 wherein the agent to favor partitioning is a multivalent, polycationic agent.

75. The method of claim 69 wherein the nonpolar organic solvent is selected from the group consisting of methylene chloride, chloroform, ethyl acetate, tetrahydrofuran, hexafluoroisopropanol, and hexafluoroacetone sesquihydrate.

76. The method of claim 69 wherein the semipolar organic solvent is selected from the group consisting of dimethylacetamide, dimethylsulfoxide, dimethylformamide, dioxane, and acetone.

77. The method of claim 69 comprising the further step of lyophilizing the nanoparticles.

78. The method of claim 77 wherein the lyophilizing step comprises subjecting the nanoparticles to -60° C under 100 millitorr vacuum for 48 hours.

79. The method of claim 77 further comprising the step of sterilizing the nanoparticles.

80. The method of claim 79 wherein the step of sterilizing comprises subjecting the nanoparticles to a sterilizing radiation.

81. The method of claim 69 wherein, in the step of sonicating, the energy sufficient form a stable emulsion is in the range of 35-65 Watts.

82. The method of claim 77 comprising the further step of modifying the surface of the resulting nanoparticles.

83. The method of claim 82 wherein the step of modifying the surface of the resulting nanoparticles comprises adsorbing at least one surface modifying agent to the nanoparticles.

84. The method of claim 83 wherein the step of adsorbing comprises the steps of suspending the nanoparticles in a solution of the at least one surface modifying agent and freeze-drying the suspension to produce a coating on the nanoparticles.

85. The method of claim 82 wherein the step of modifying the surface comprises epoxy-derivatization.

86. The method of claim 85 comprising the further step of reacting the epoxy-activated nanoparticles with reactive groups on one or more bioactive agents or surface modifying agents, which reactive groups may be amino, anhydrides, carboxyl, hydroxyl, phenol, or sulfhydryl.

87. The method of claim 82 wherein the step of modifying the surface comprises incorporating the at least one surface modifying agent in the polymer matrix.

Protein/Peptide Agents

88. A method of making a sustained release drug delivery system for water-soluble protein/peptide-containing bioactive agents comprising the steps of:

(a) dissolving the water-soluble protein/peptide-containing bioactive agent in an aqueous solution to form a first aqueous phase;

(b) dissolving the polymer in a nonpolar organic solvent;

(c) adding the first aqueous phase to the organic polymer solution to form a primary emulsion;

(d) emulsifying the primary emulsion into an aqueous solution of an emulsifying agent to form a water-in-oil-in-water emulsion;

(e) evaporating the organic solvent from the water-in-oil-in-water emulsion; and

(f) separating resulting nanoparticles the remaining aqueous phase.

89. The method of claim 88 comprising the further step of lyophilizing the nanoparticles.

90. The method of claim 88 wherein step (c) comprises sonicating the protein-containing aqueous solution and the organic solution with energy sufficient to form a stable primary emulsion.

91. The method of claim 88 wherein the aqueous solution of an emulsifying agent is an aqueous solution of an emulsifying agent for making water-in-oil emulsions selected from the group consisting of sorbitan esters of fatty acids, fatty alcohols, fatty acids, and glycerol esters of fatty acids.

92. The method of claim 88 wherein the aqueous solution of an emulsifying agent is an aqueous solution of an emulsifying agent for making oil-in-water emulsions selected from the group consisting of polyoxyethylene ethers of fatty alcohols, polyoxyl fatty acid esters, polyoxyethylene glycols of fatty acids.

93. The method of claim 88 wherein the water-soluble protein-containing bioactive agent is a nucleic acid selected from the group consisting of DNA, RNA, or an oligonucleotide (sense or antisense) of DNA or RNA.

94. The method of claim 93 wherein the water-soluble protein-containing bioactive agent is nuclease-free DNA.

95. The method of claim 94 wherein the aqueous solution is nuclease-free and/or includes a calcium complexing agent.

96. The method of claim 95 wherein the aqueous solution is a buffer selected from the group of Tris-EDTA, dithizone, nitrolotriacetic acid, citrates, oxalates, tartrates, and dimercaprol.

97. The method of claim 93 further comprising the step of modifying the surface of the resulting nanoparticles.

98. The method of claim 89 wherein the step of modifying the surface of the resulting nanoparticles comprises adsorbing at least one surface modifying agent to the nanoparticles.

99. The method of claim 98 wherein the step of adsorbing comprises the steps of suspending the nanoparticles in a solution of the at least one surface modifying agent and freeze-drying the suspension to produce a coating on the nanoparticles.

100. The method of claim 89 wherein the step of modifying the surface comprises epoxy-derivatization.

101. The method of claim 89 epoxy-derivatization comprises the steps of partially hydrolyzing the nanoparticles to create reactive groups on the surface; and contacting the hydrolyzed nanoparticles with a reactive multifunctional epoxide to form epoxy-activated nanoparticles.

102. The method of claim 101 comprising the further step of reacting the epoxy-activated nanoparticles with reactive groups on one or more bioactive agents and/or surface modifying agents.

103. The method of claim 89 wherein the step of modifying the surface comprises incorporating the at least one surface modifying agent in the polymer matrix.

Epoxy-Derivatization Method

104. A method of modifying the surface of a polymer of the type having a reactive end group, the method comprising the steps of:

contacting the polymer with a multifunctional epoxide compound in the presence of a catalyst to form an epoxide-coupled polymer; and

reacting the epoxide-coupled polymer with a bioactive agent having at least one functional group thereon which reacts with epoxide groups to covalently link the bioactive agent to the polymer.

105. The method of claim 104 wherein the polymer has at least one reactive end group selected from the group consisting of amino, anhydrides, carboxyl, hydroxyl, phenol, or sulfhydryl.

106. The method of claim 105 wherein the polymer is selected from the group consisting of polyesters, such as polylactide, polyglycolides, polylactic polyglycolic copolymer, and polycaprolactone.

107. The method of claim 106 wherein the polymer is a poly-lactide-co-poly-glycolide.

108. The method of claim 104 wherein the polymer is at least partially hydrolyzed prior to contacting the polymer with the multifunctional epoxide compound.

109. The method of claim 104 wherein the epoxide compound is an epoxide, a polyepoxide compounds, or an epoxy resin.

110. The method of claim 109 wherein the epoxide compound is selected from the group consisting of 1,2-epoxides, 1,2-propylene oxides, butane and ethane di-glycidyl ethers, erythritol anhydride, polyfunctional polyglycerol polyglycidyl ethers, and epichlorhydrin.

111. The method of claim 104 wherein the catalyst is selected from the group consisting of tertiary amines, guanidine, imidazole, boron trifluoride adducts, such as boron trifluoride-monoethylamine, trace metals, bisphosphonates, and ammonium complexes of the type $PhNH_3 + AsF_6$.

112. The method of claim 104 wherein the catalyst is suitable for photoinitiation.

113. The method of claim 112 wherein the catalyst is selected from the group consisting of titanium tetrachloride and ferrocene, zirconocene chloride, carbon tetrabromides and iodoform. 111.

114. The method of claim 104 wherein the bioactive agent has at least one reactive end group selected from the group consisting of amino, anhydrides, carboxyl, hydroxyl, phenol, or sulfhydryl.

Method of Making PCL-Embodiments

115. A method of making block copolymers having hydrophilic and hydrophobic segments, the method comprising the steps of:

(a) dissolving a first polymer-diol in an organic solvent;

(b) adding a multifunctional epoxide in excess to the dissolved first polymer-diol so that one of the epoxide groups of the multifunctional epoxide reacts with hydroxyl groups on the ends of the first polymer-diol to form an epoxide end-capped first polymer (block A);

(c) adding an excess of a second polymer-diol (block B) to the epoxide end-capped first polymer block A to form a hydroxyl-terminated BAB-type triblock copolymer.

116. The method of claim 115 wherein there is provided the further step of expanding the molecular weight of a polymer-diol, prior to use in step (a), by reacting an excess of the polymer-diol with a polyfunctional epoxide.

117. The method of claim 115 wherein the multifunctional epoxide is selected from the group consisting of 1,2-epoxides, 1,2-propylene oxides, butane and ethane di-glycidyl ethers, erythritol anhydride, polyfunctional polyglycerol polyglycidyl ethers, and epichlorhydrin.

118. The method of claim 115 wherein the first polymer-diol is a hydrophobic polymer-diol.

119. The method of claim 118 wherein the hydrophobic polymer-diol is selected from the group consisting of polycaprolactone, polylactides, polyglycolides, and polylactic-polyglycolic acid copolymer.

120. The method of claim 115 wherein the second polymer-diol is a hydrophilic polymer-diol.

121. The method of claim 120 wherein the hydrophilic polymer-diol is selected from the group consisting of polyethylene glycol, polaxomers, and poly(propylene oxide).

122. The method of claim 115 wherein the first polymer-diol is a hydrophilic polymer-diol.

123. The method of claim 122 wherein the second polymer-diol is a hydrophobic polymer-diol.

124. The method of claim 115 comprising the further step of reacting the BAB-type triblock copolymer with a multifunctional epoxide to form an epoxide end-capped BAB-type triblock copolymer.

125. The method of claim 124 comprising the further step of reacting the epoxide end-capped BAB-type triblock copolymer with a bioactive agent having at least one functional group thereon which reacts with epoxide groups to covalently attach the bioactive agent to the epoxide end-capped BAB-type triblock copolymer.

126. The method of claim 115 wherein steps (b) and (c) are repeated to form multiblock copolymers.

127. The method of claim 126 comprising the further step of reacting the multiblock copolymer with a multifunctional epoxide to form an epoxide end-capped multiblock polymer.

128. The method of claim 127 wherein the reactive multiblock polymer is washed or reacted to block further epoxide reactivity.

Composition Claims to Multiblock Copolymers

129. Multiblock copolymers having hydrophobic and hydrophilic segments connected by epoxy linkages and being hydroxy-terminated or epoxide-terminated and having a molecular weight between about 6,000 to 100,000 as measured by gel permeation chromatography and intrinsic viscosity.

130. The multiblock copolymer of claim 129 wherein the hydrophobic segment is selected from the group consisting of polycaprolactone, polylactides, polyglycolides, polylactic-polyglycolic acid copolymer, biodegradable polyurethanes, polyanhydrides, and polyamino acids.

131. The multiblock copolymer of claim 129 wherein the hydrophilic segment is a polyether selected from the group consisting of polyethylene glycol, polaxomers, and poly(propylene oxide).

132. The polymers of claim 129 which are ABA, BAB, multiblock $(AB)_n$ or $(BA)_n$ type polymers, and combinations thereof, wherein the A block is polycaprolactone and the B block is selected from the group consisting of polyethylene glycol, poloxamers, and poly(propylene oxide).

133. The polymers of claim 129 wherein a hydrophobic and/or hydrophilic segment is expanded, *i.e.*, multiple molecules are linked together by epoxy linkages.

134. Hydroxy-terminated poly (ϵ-caprolactone)-polyether polymers having alternating hydrophobic polycaprolactone segments and hydrophilic polyether segments connected by epoxy linkages.

135. The block copolymer which is HO-PEG-EX252-PCL-EX252-PCL-EX252-PEG-OH.

136. The block copolymer which is HO-F68-EX252-PCL-EX252-PCL-F68-OH.

137. The block copolymer which is HO-PCL-EX252-F68-EX252-PCL-OH.

138. The block copolymer which is HO-PCL-EX252-PEG-EX252-PCL-OH.

139. The block copolymer which is HO-PCL-EX252-PPO-EX252-PCL-OH.

140. The multiblock copolymer of claim 125 comprising a nanoparticle.

Methods of Use Embodiments

Restenosis

141. A method of preventing restenosis following vascular damage as a result of an interventional procedure or disease:

injecting nanoparticles comprising a biocompatible, biodegradable polymer core having an average diameter of less than about 300 nm, the nanoparticles having associated or incorporated therewith at least one bioactive agent and/or at least one surface modifying agent, under pressure into the wall of the vessel preceding, during, or subsequent to the damaging interventional procedure.

142. The method of preventing restenosis of claim 141 wherein the pressure is at least 1 atm and preferably between 3-6 atm.

143. The method of preventing restenosis of claim 141 wherein the step of injecting is accomplished with a catheter.

144. The method of preventing restenosis of claim 141 comprising the further step of inducing an osmotic shock to the wall of the vessel with a hypertonic solution prior to or contemporaneously with the step of injecting nanoparticles.

145. The method of preventing restenosis of claim 141 wherein the biocompatible, biodegradable polymer is a synthetic polymer selected from the group consisting of polyesters, polyethers, polyanhydrides, polyalkylcyanoacrylates, polyacrylamides, poly(orthoesters), polyphosphazenes, polyamino acids, and biodegradable polyurethanes.

146. The method of preventing restenosis of claim 141 wherein the biocompatible, biodegradable polymer is a naturally-derived polymer selected from the group consisting of acacia, chitosan, gelatin, dextrans, albumins, and alginates/starch.

147. The method of claim 141 wherein the bioactive agent is selected from the group consisting of smooth muscle inhibitors, receptor blockers for contractile agonist, inhibitors of the sodium/hydrogen antiporter, protease inhibitors, nitrovasodilators, phosphodiesterase inhibitors, phenothiazines, growth factor receptor antagonists, anti-mitotic agents, immunosuppressive agents, antisense oligonucleotides, and protein kinase inhibitors.

148. The method of preventing restenosis of claim 147 wherein the bioactive agent is a cytochalasin.

149. The method of preventing restenosis of claim 141 wherein the nanoparticles further includes at least one surface modifier.

150. The method of preventing restenosis of claim 149 the surface modifier is selected from the group consisting of the surface modifying agent is selected from the group consisting of one or more synthetic polymers, biopolymers, low molecular weight oligomers, natural products, and surfactants.

151. The method of preventing restenosis of claim 150 wherein the surface modifier is fibrinogen and/or DMAB.

152. The method of preventing restenosis of claim 141 wherein the nanoparticles are suspended in a suspending media suitable for intravascular administration in a concentration range from about 0.1 mg/ml or less to 300 mg/ml, and preferably in the range of 5 to 30 mg/ml.

Bone Therapy

153. A method of transferring a nucleic acid segment into bone progenitor cells comprising: injecting into a tissue site having bone progenitor cells nucleic acid-containing nanoparticles, the nanoparticles comprising a biocompatible, biodegradable polymer core having an average diameter of less than about 300 nm, and having associated or incorporated therewith at least one nucleic acid and/or at least one surface modifying agent.

154. The method of claim 153 wherein the nucleic acid is an osteotropic gene or gene segment selected from the group consisting of bone morphogenic proteins (BMP2 and 4 and others), phosphoproteins, osteonectin, osteopontin, bone sialoprotein, vitamin-k dependent proteins, glycoproteins, and collagen (at least I and II).

155. The method of claim 153 wherein the surface modifying agent is selected from the group consisting of one or more synthetic polymers, biopolymers, low molecular weight oligomers, natural products, and surfactants.

156. The method of claim 153 wherein the injected nanoparticles are carried in a suspending medium gels after application to the region of injection.

157. The method of claim 156 wherein the suspending medium which gels is selected from the group consisting of poloxamers, Type I collagen or procollagen, hydrogels, cyanoacrylates, and fibrin glue.

158. The method of claim 156 wherein the suspending medium further includes at least one osteoconductive salt.

FIG. 1

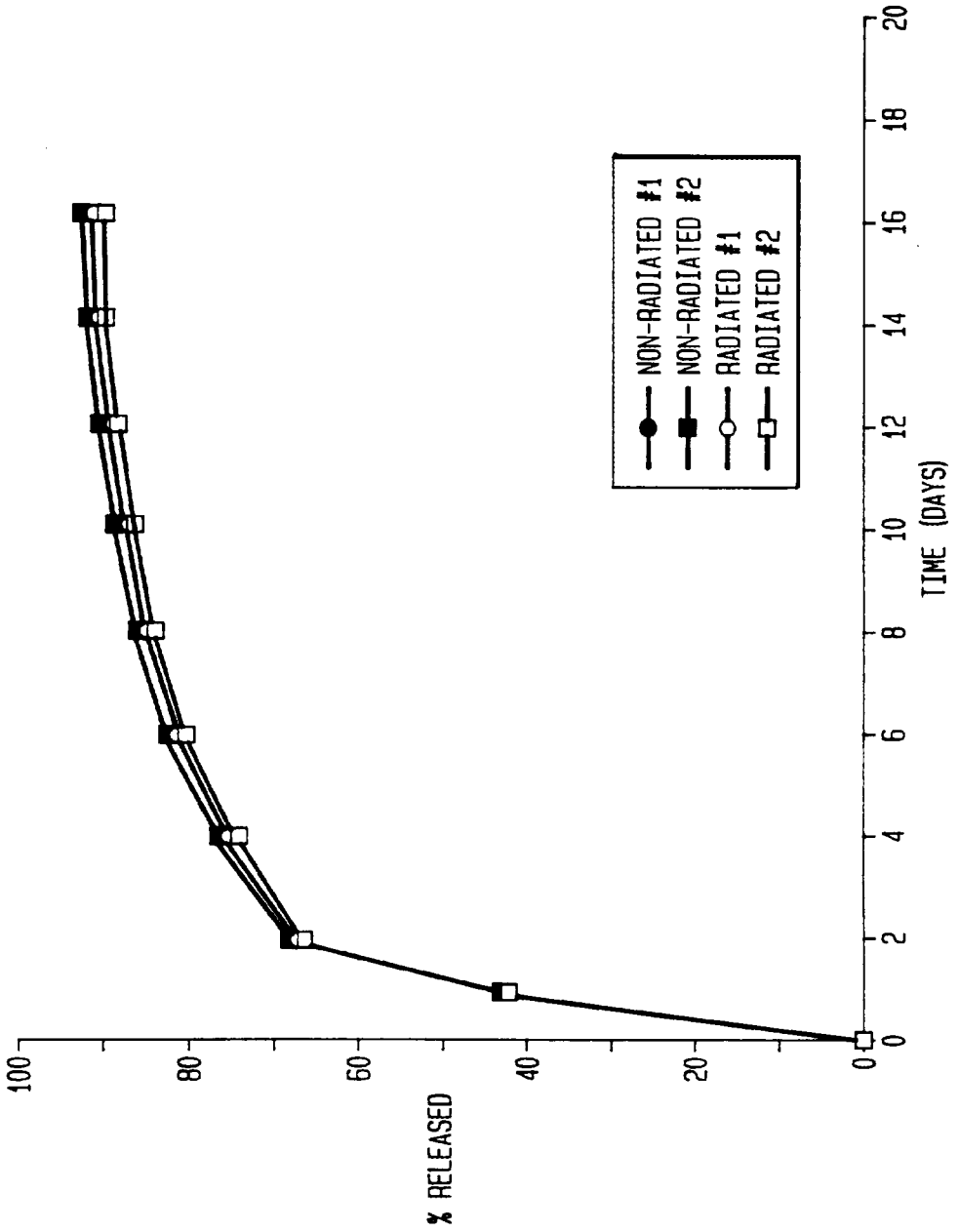

NON-RADIATED #1
NON-RADIATED #2
RADIATED #1
RADIATED #2
% RELEASED
TIME (DAYS)
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16
18
20

FIG. 2

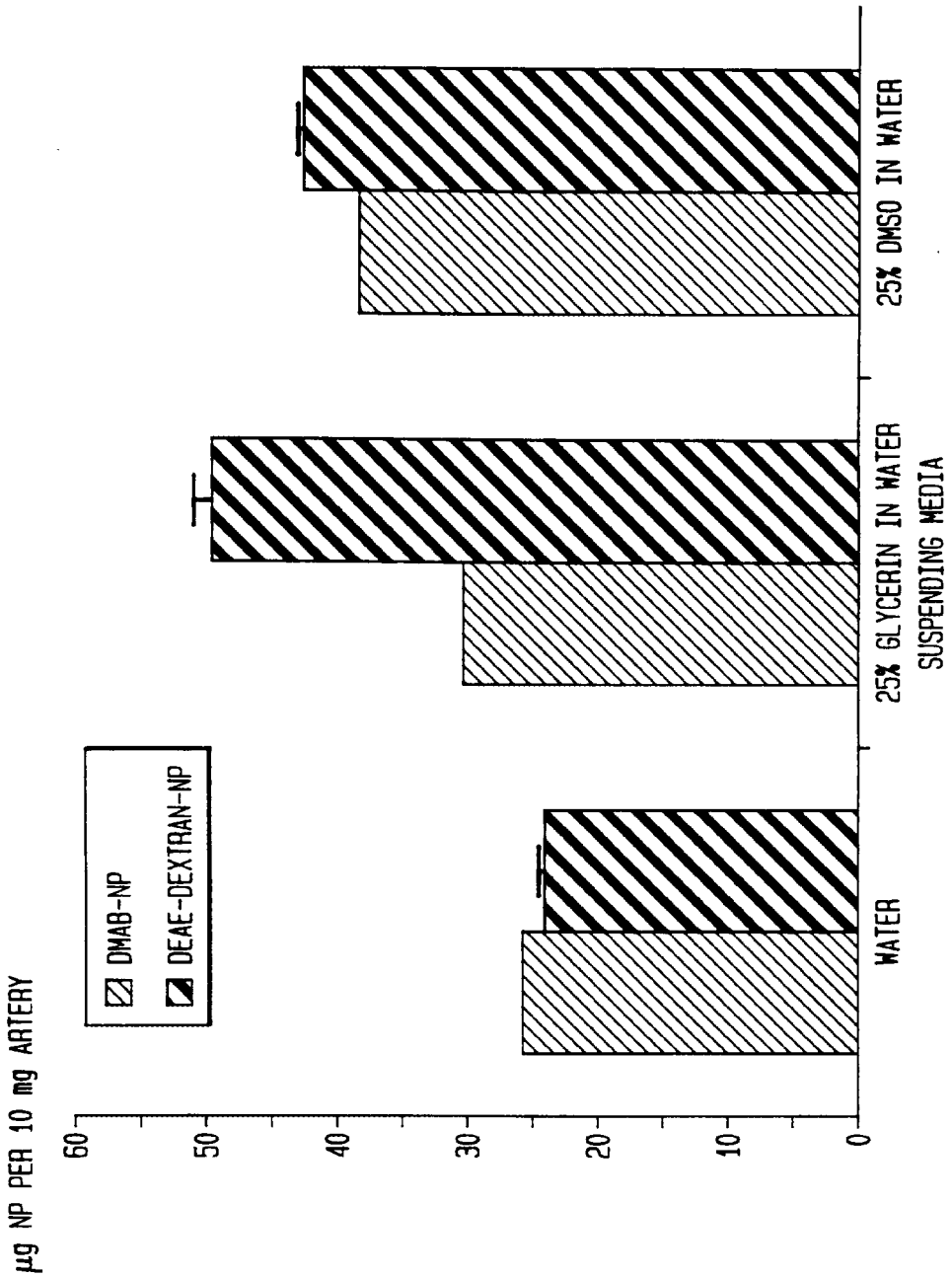

µg NP PER 10 mg ARTERY
60
50
40
30
20
10
0
DMAB-NP
DEAE-DEXTRAN-NP
WATER
25% GLYCERIN IN WATER
25% DMSO IN WATER
SUSPENDING MEDIA

FIG. 3

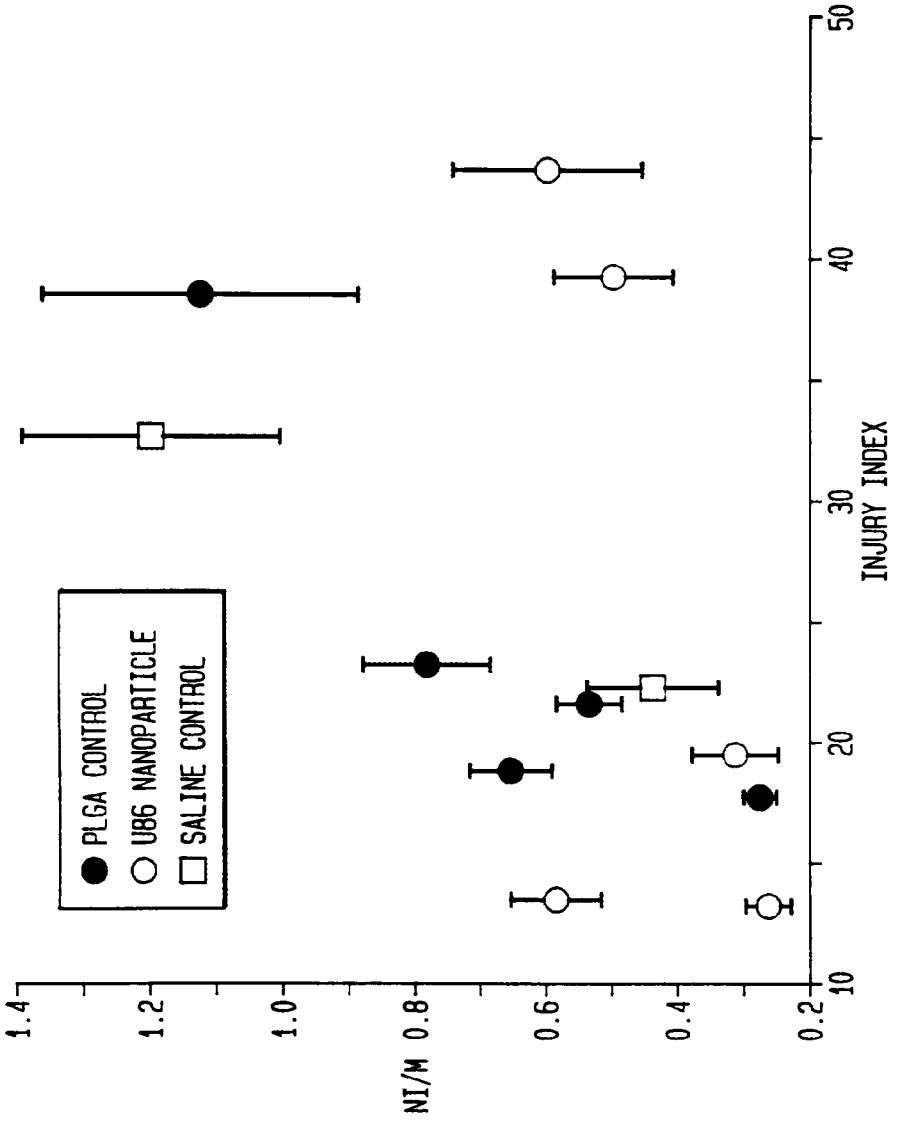

PLGA CONTROL
U86 NANOPARTICLE
SALINE CONTROL
NI/M
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
INJURY INDEX

FIG. 4

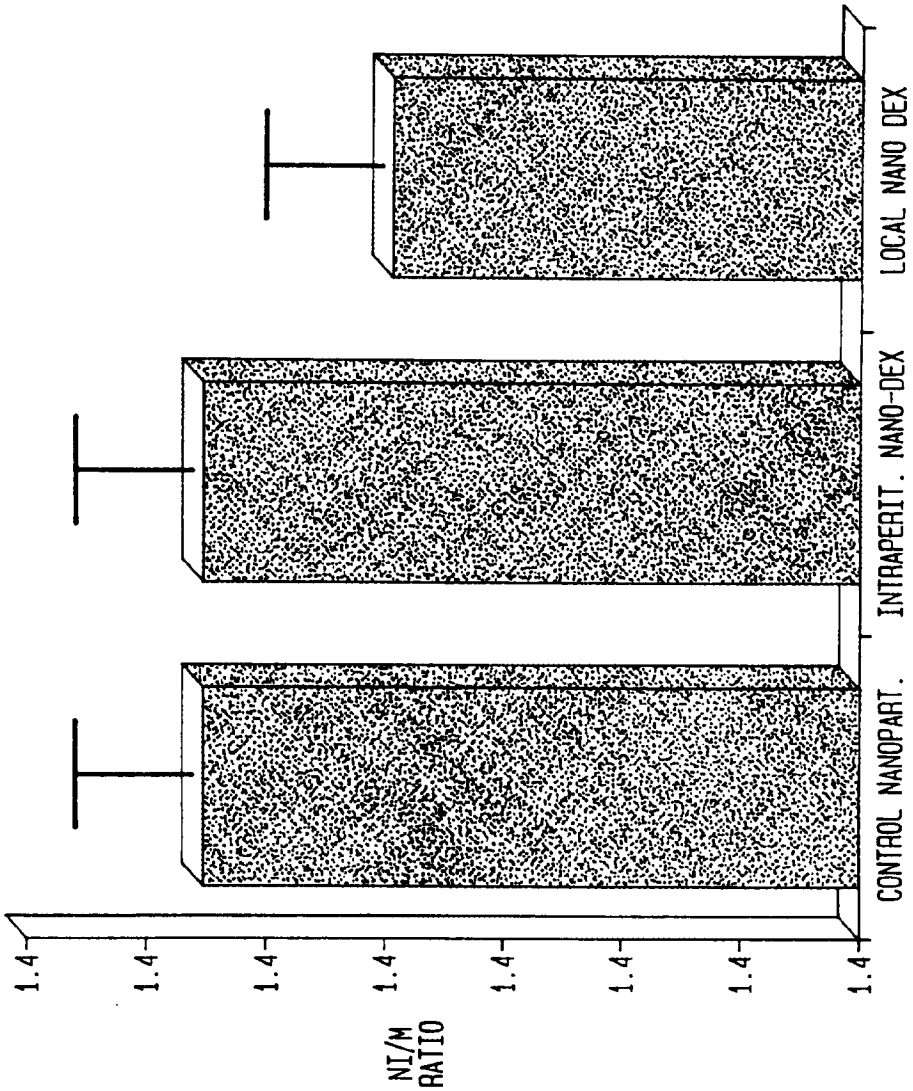

NI/M RATIO
1.4
1.4
1.4
1.4
1.4
1.4
1.4
1.4
CONTROL NANOPART.
INTRAPERIT. NANO-DEX
LOCAL NANO DEX

FIG. 5

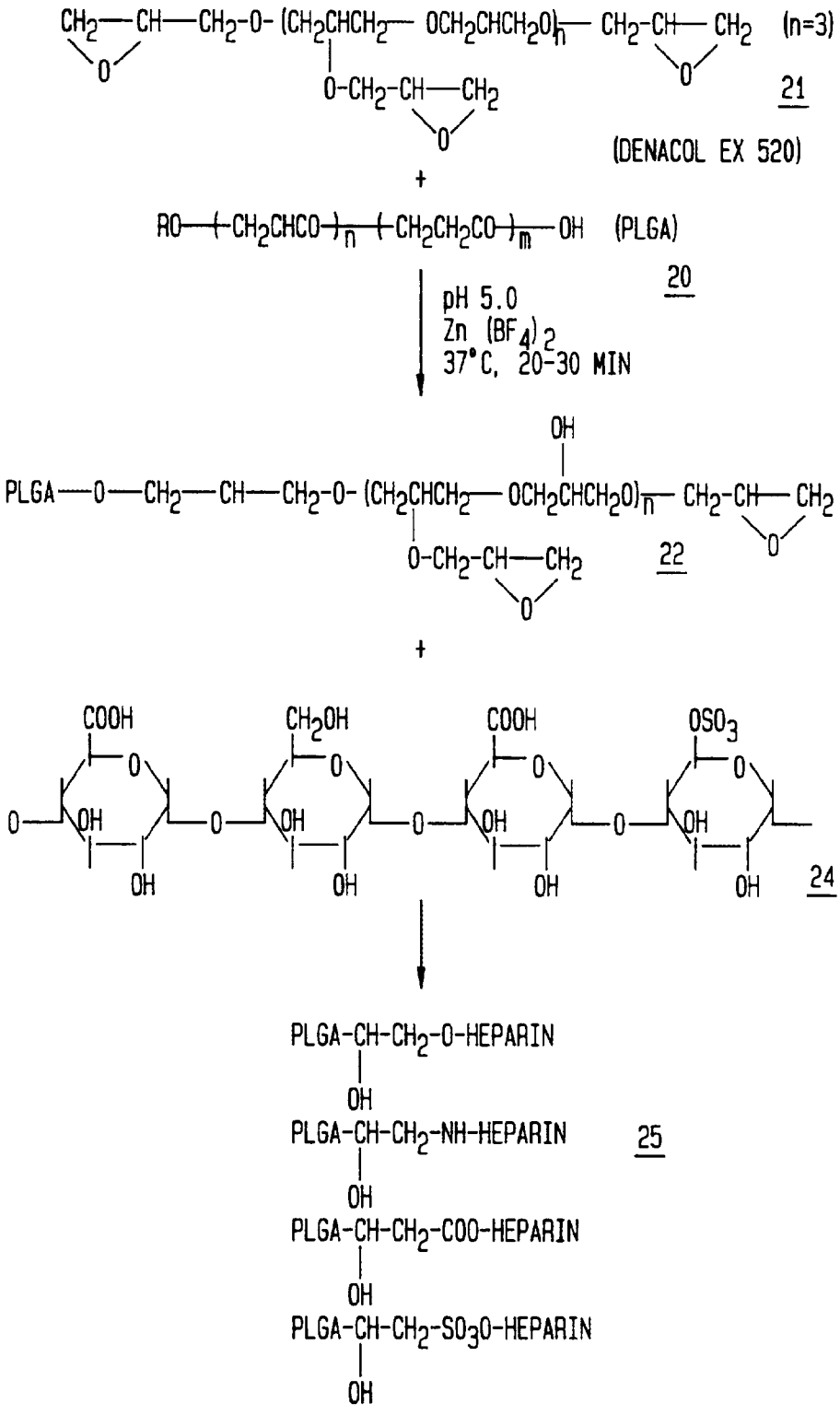

(n=3)
21
(DENACOL EX 520)
(PLGA)
20
pH 5.0
Zn (BF4)2
37°C, 20-30 MIN
22
24
PLGA-CH-CH2-O-HEPARIN
OH
PLGA-CH-CH2-NH-HEPARIN
25
OH
PLGA-CH-CH2-COO-HEPARIN
OH
PLGA-CH-CH2-SO3O-HEPARIN
OH

FIG. 6

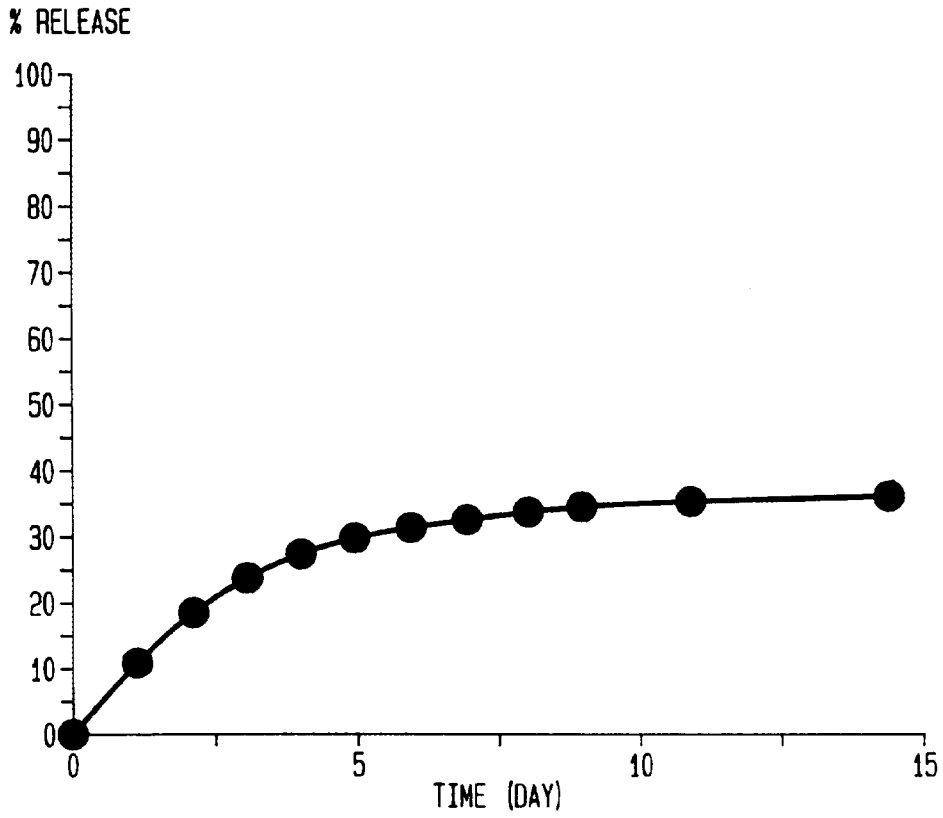


FIG. 7

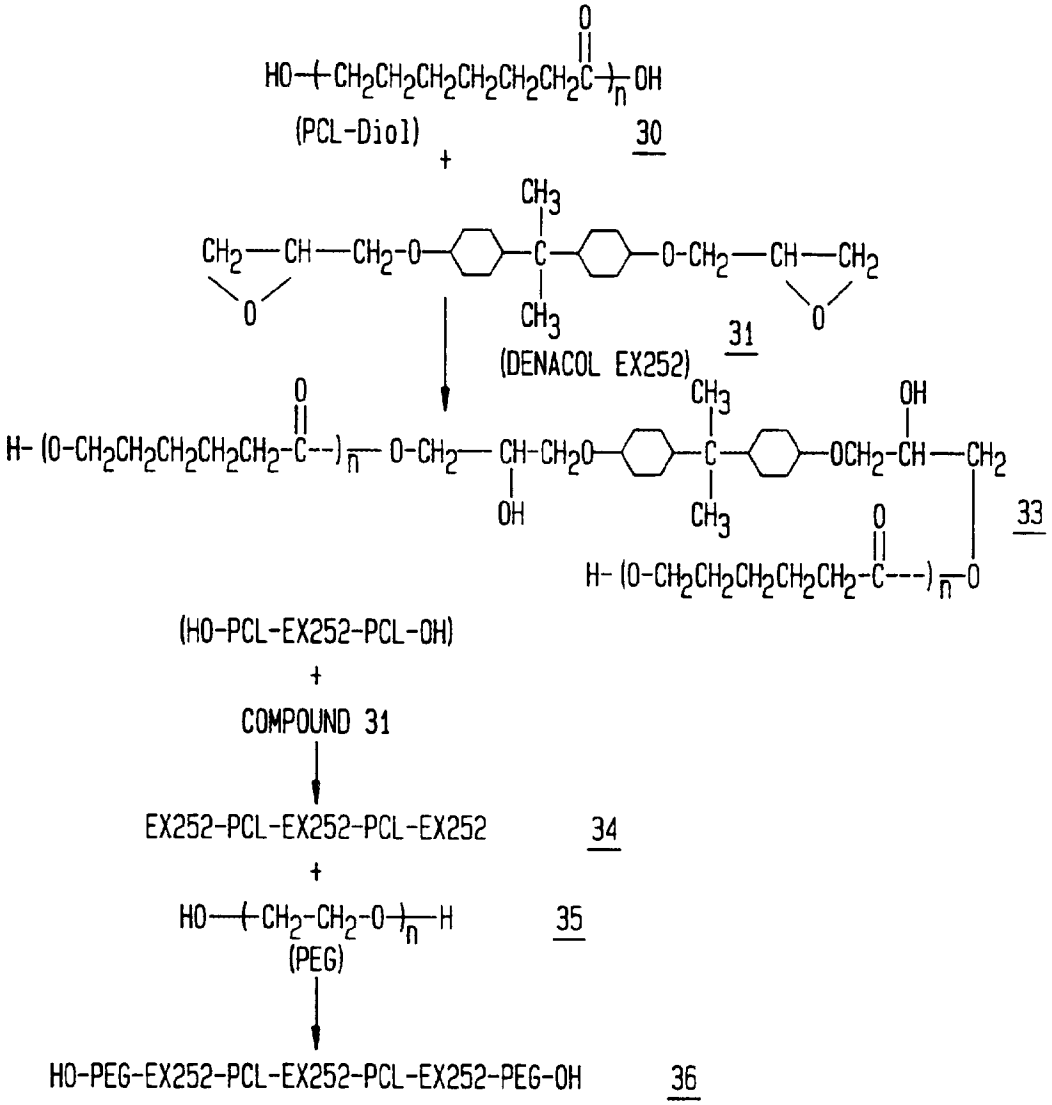
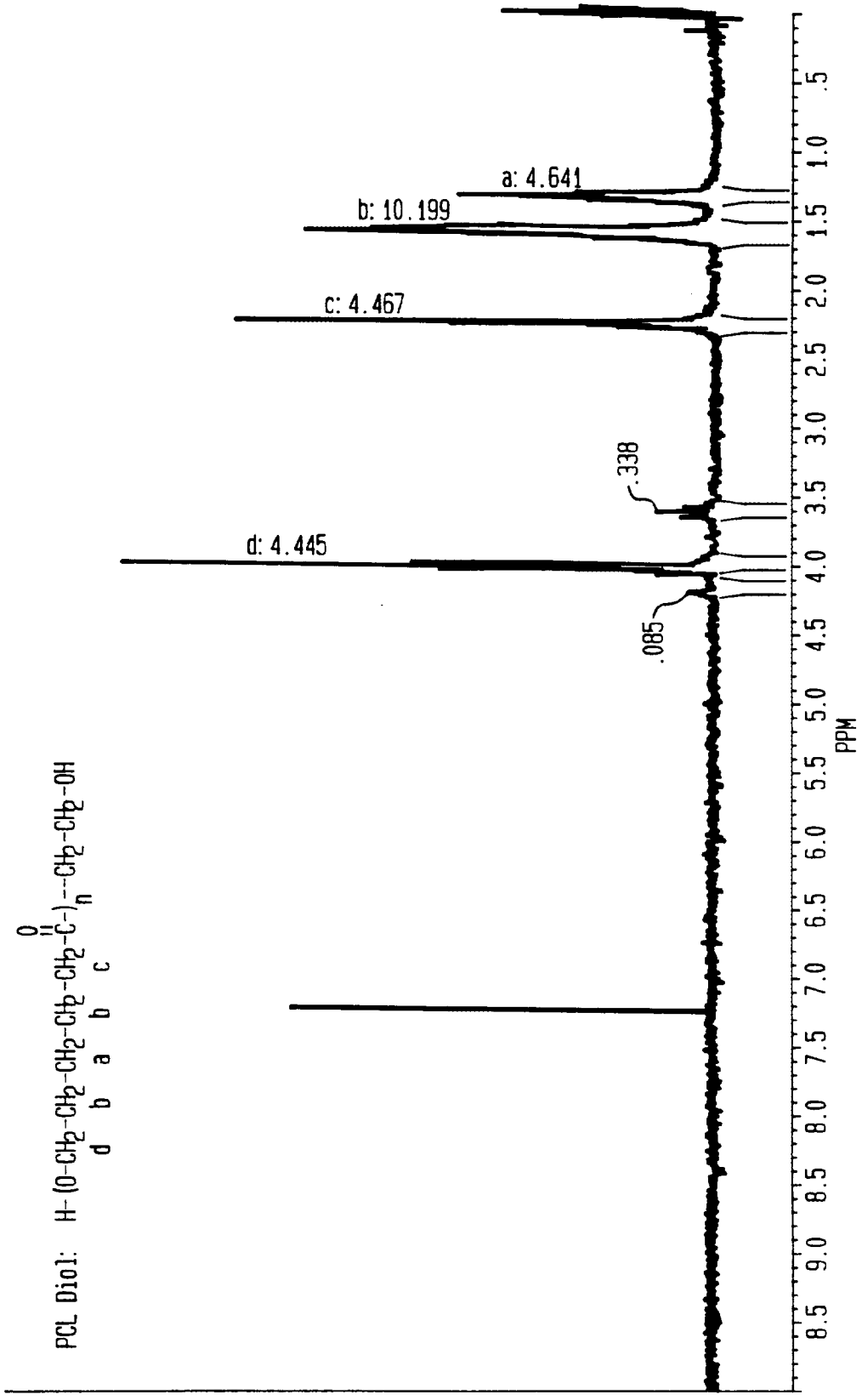

HO-(-CH2CH2CH2CH2CH2CH2C-)n-OH
(PCL-Diol)
30
+
CH2—CH—CH2-O
O
CH3
C
CH3
O-CH2—CH—CH2
O
31
(DENACOL EX252)
H-(O-CH2CH2CH2CH2CH2-C--)n-O-CH2-CH-CH2O
OH
CH3
C
CH3
OCH2-CH—CH2
OH
O
33
H-(O-CH2CH2CH2CH2CH2-C---)n-O
(HO-PCL-EX252-PCL-OH)
+
COMPOUND 31
EX252-PCL-EX252-PCL-EX252
34
+
HO-(-CH2-CH2-O-)n-H
(PEG)
35
HO-PEG-EX252-PCL-EX252-PCL-EX252-PEG-OH
36

FIG. 8


PCL Diol: H-(O-CH2-CH2-CH2-CH2-CH2-C(=O)-)n-CH2-CH2-OH
d b a b c
a: 4.641
b: 10.199
c: 4.467
.338
d: 4.445
.085
PPM

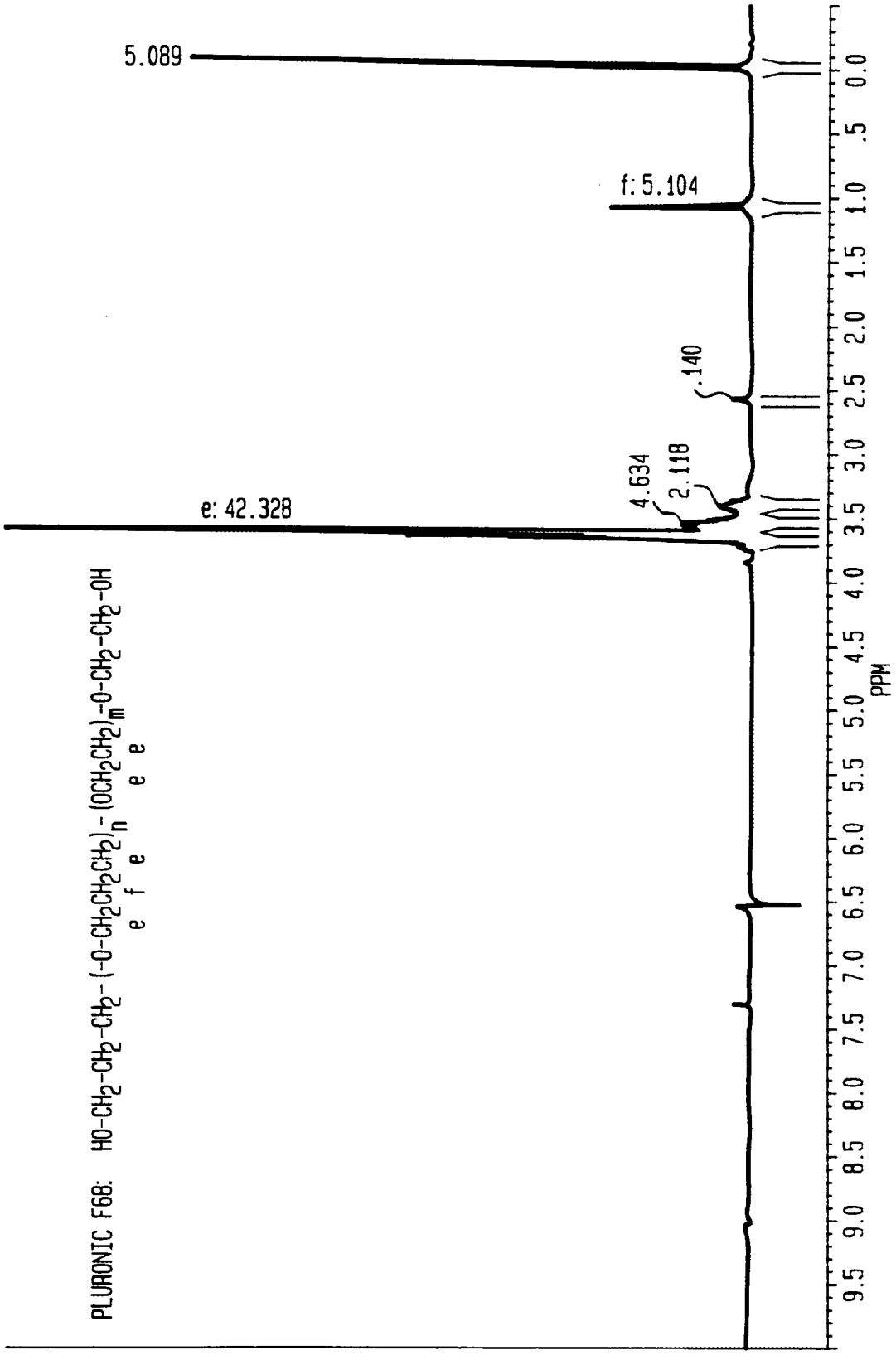

5.089
f: 5.104
.140
4.634
2.118
e: 42.328
PLURONIC F68: HO-CH2-CH2-CH2-(-O-CH2CH2CH2)n-(OCH2CH2)m-O-CH2-CH2-OH
e f e e e
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 .5 0.0
PPM

FIG. 9

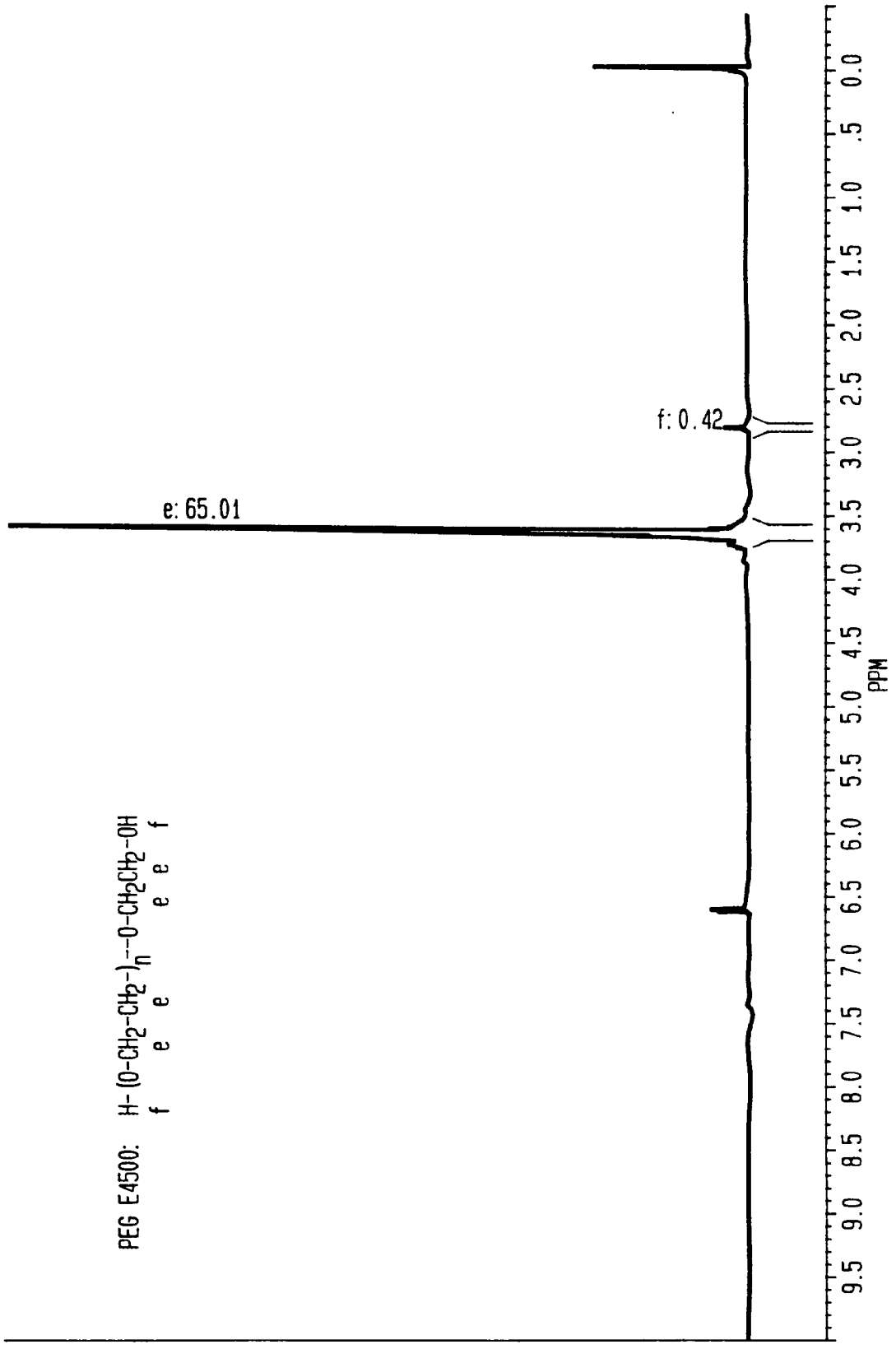
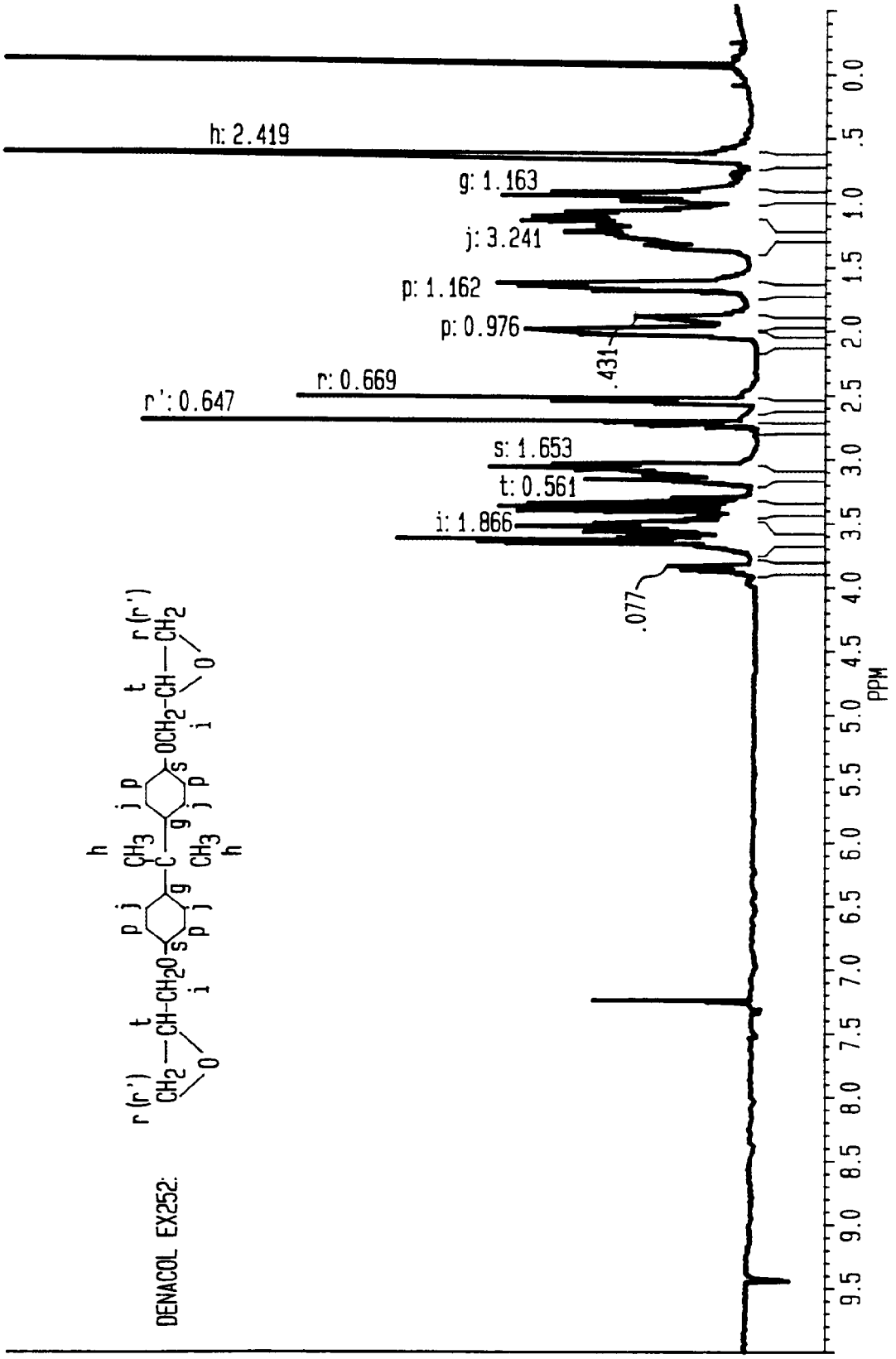

f: 0.42
e: 65.01
PEG E4500: H-(O-CH2-CH2-)n-O-CH2CH2-OH
f e e e f
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 .5 0.0
PPM

FIG. 10

FIG. 11


h: 2.419
g: 1.163
j: 3.241
p: 1.162
p: 0.976
.431
r: 0.669
r': 0.647
s: 1.653
t: 0.561
i: 1.866
.077
0.0
.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
PPM
DENACOL EX252:

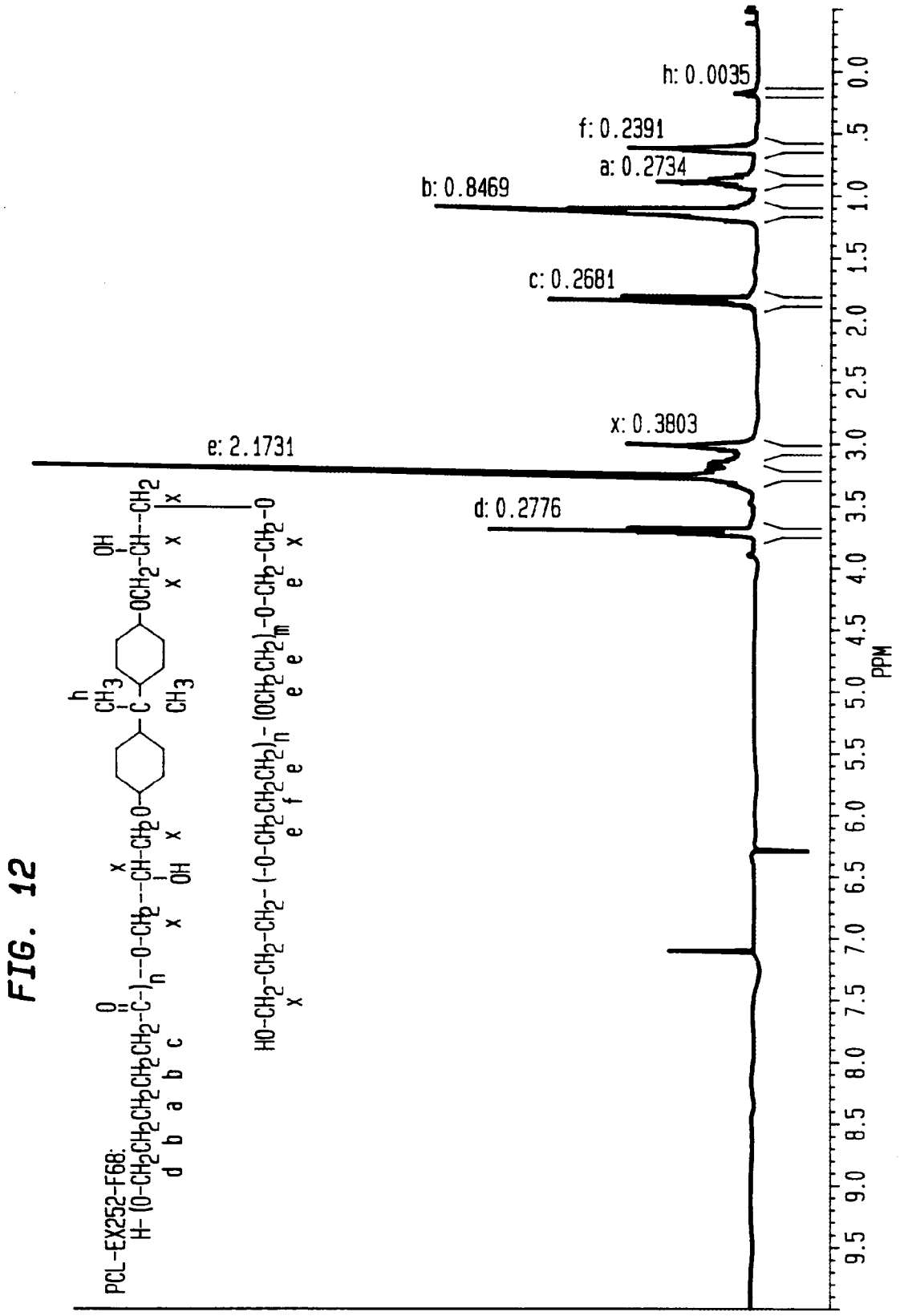

h: 0.0035
f: 0.2391
a: 0.2734
b: 0.8469
c: 0.2681
x: 0.3803
e: 2.1731
d: 0.2776
PPM
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 .5 0.0
PCL-EX252-F68:
H-(O-CH2CH2CH2CH2CH2CH2C-)n-O-CH2--CH-CH2O
d b a b c x x OH
HO-CH2-CH2-CH2-(-O-CH2CH2CH2)-(OCH2CH2)m-O-CH2-CH2-O
x e f e n e e x
h
CH3
C
CH3
OCH2-CH--CH2
x x OH x x

FIG. 12

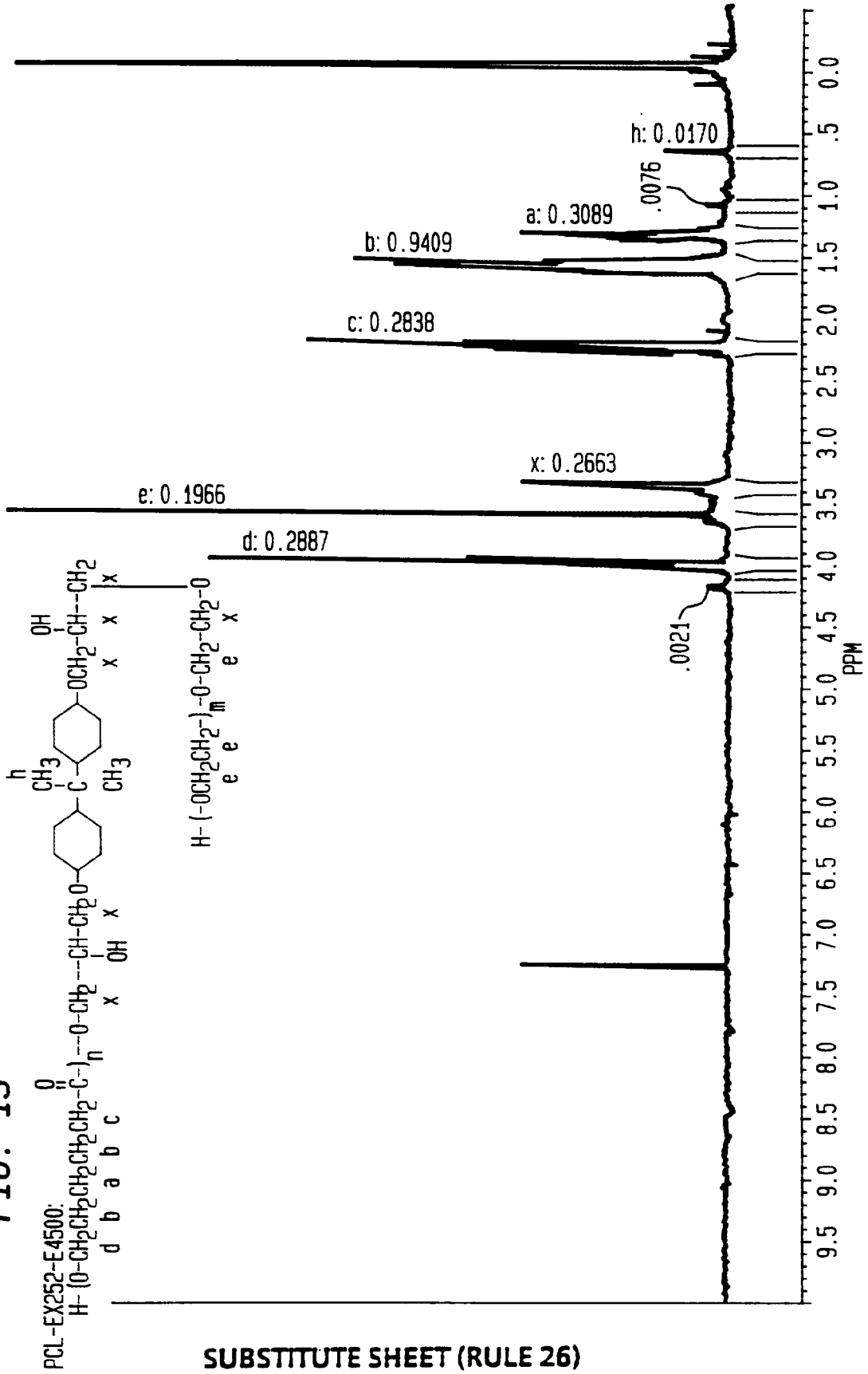

h: 0.0170
.0076
a: 0.3089
b: 0.9409
c: 0.2838
x: 0.2663
e: 0.1966
d: 0.2887
.0021
PPM
0.0
.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
PCL-EX252-E4500:
H-(O-CH2CH2CH2CH2CH2CH2-C-)n-O-CH2-CH-CH2O
H-(-OCH2CH2-)m-O-CH2-CH2-O
OH
OCH2-CH-CH2
CH3
CH3

FIG. 13

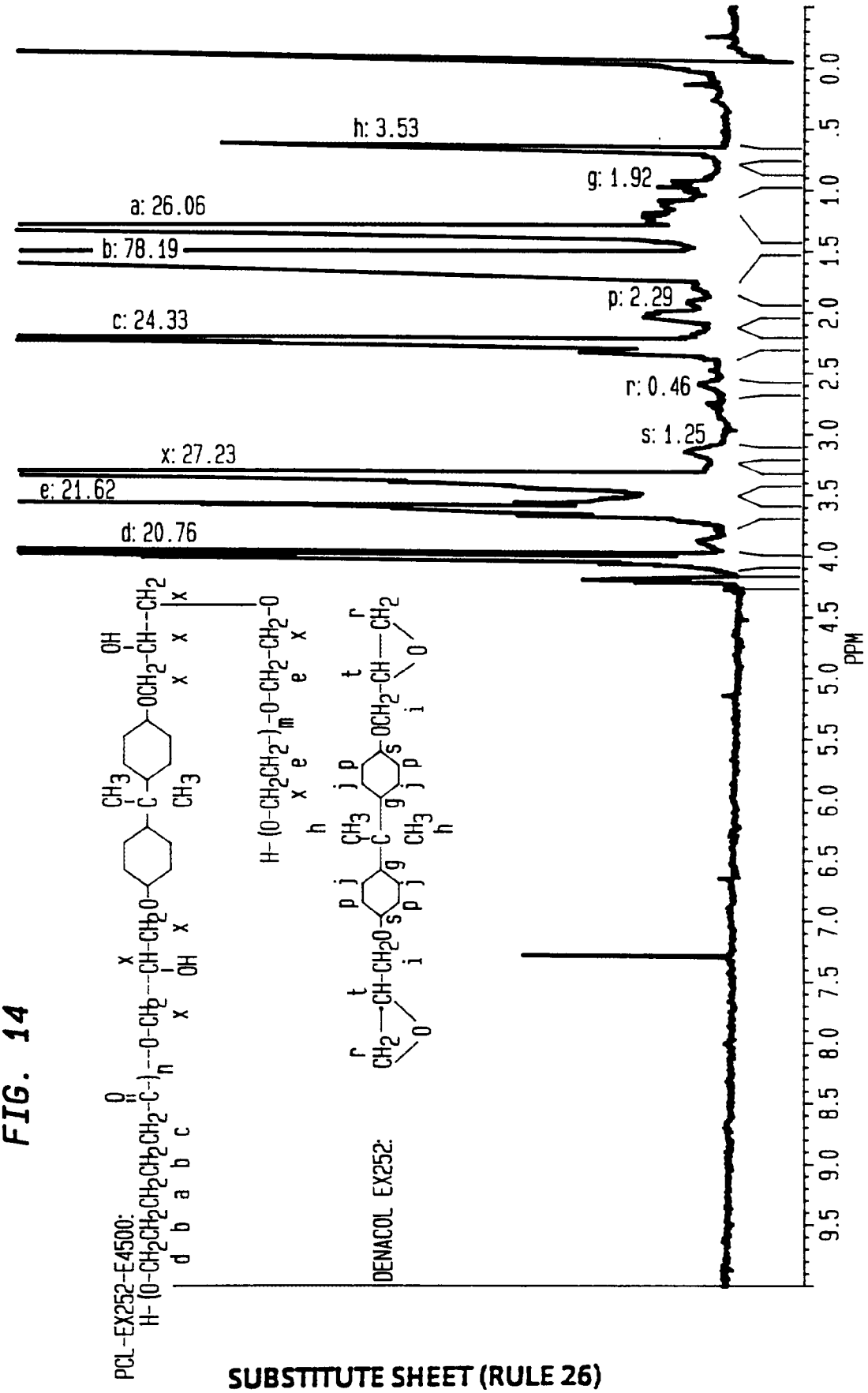

h: 3.53
g: 1.92
a: 26.06
b: 78.19
p: 2.29
c: 24.33
r: 0.46
s: 1.25
x: 27.23
e: 21.62
d: 20.76
PPM
PCL-EX252-E4500:
DENACOL EX252:

FIG. 14

FIG. 15

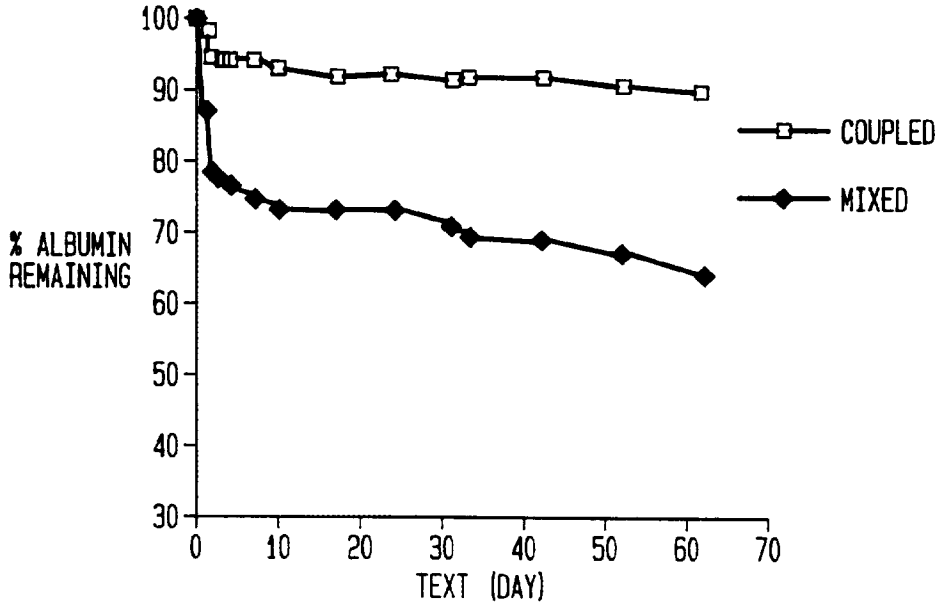

100
90
80
70
60
50
40
30
% ALBUMIN REMAINING
0
10
20
30
40
50
60
70
TEXT (DAY)
COUPLED
MIXED

FIG. 16A

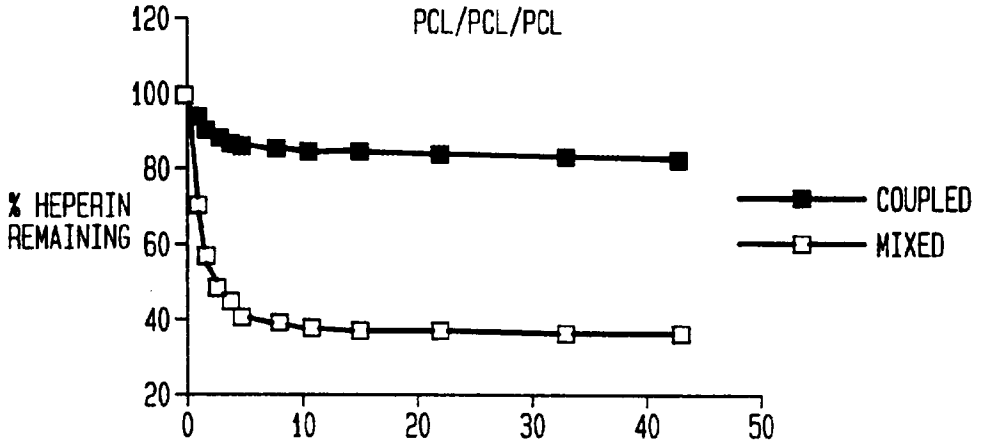


FIG. 16B

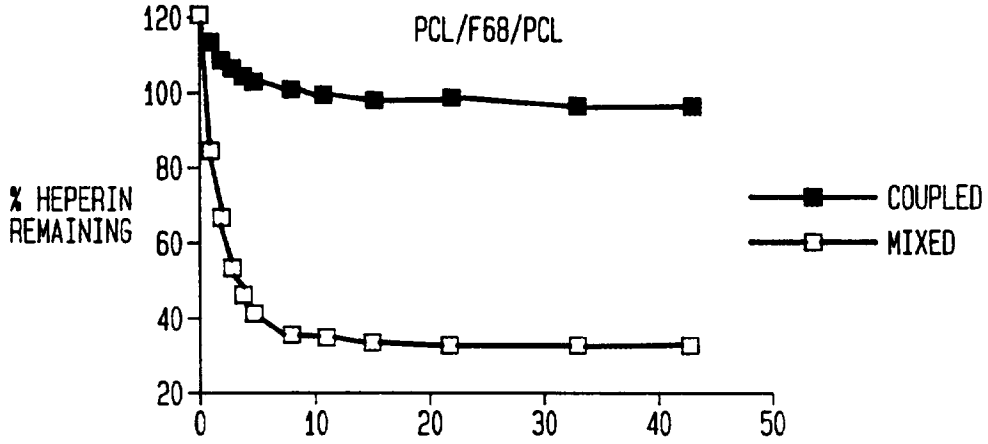


FIG. 16C

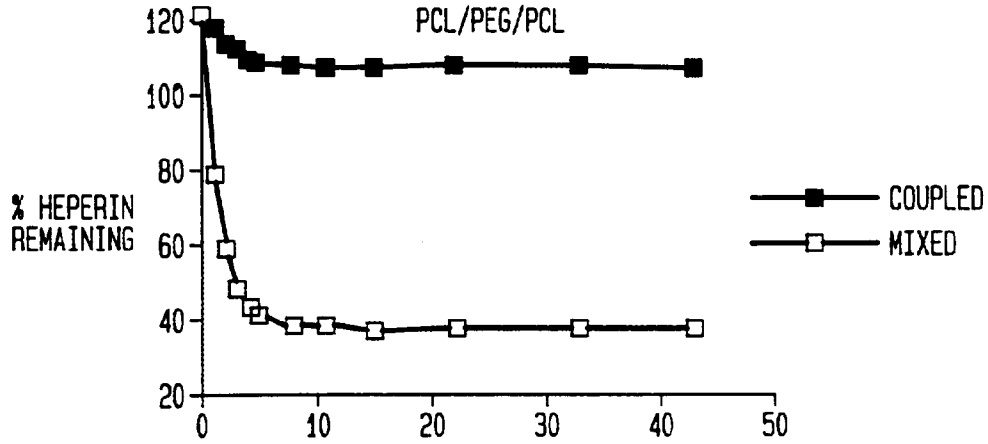


FIG. 17

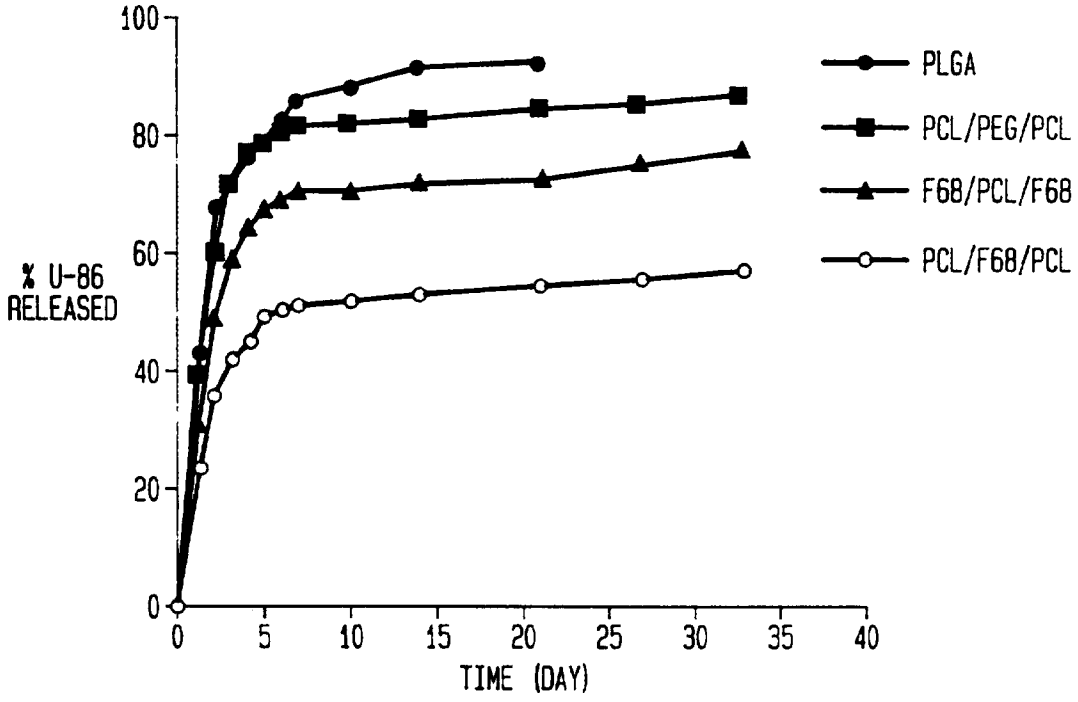

100
80
60
40
20
0
% U-86 RELEASED
0
5
10
15
20
25
30
35
40
TIME (DAY)
PLGA
PCL/PEG/PCL
F68/PCL/F68
PCL/F68/PCL

FIG. 18

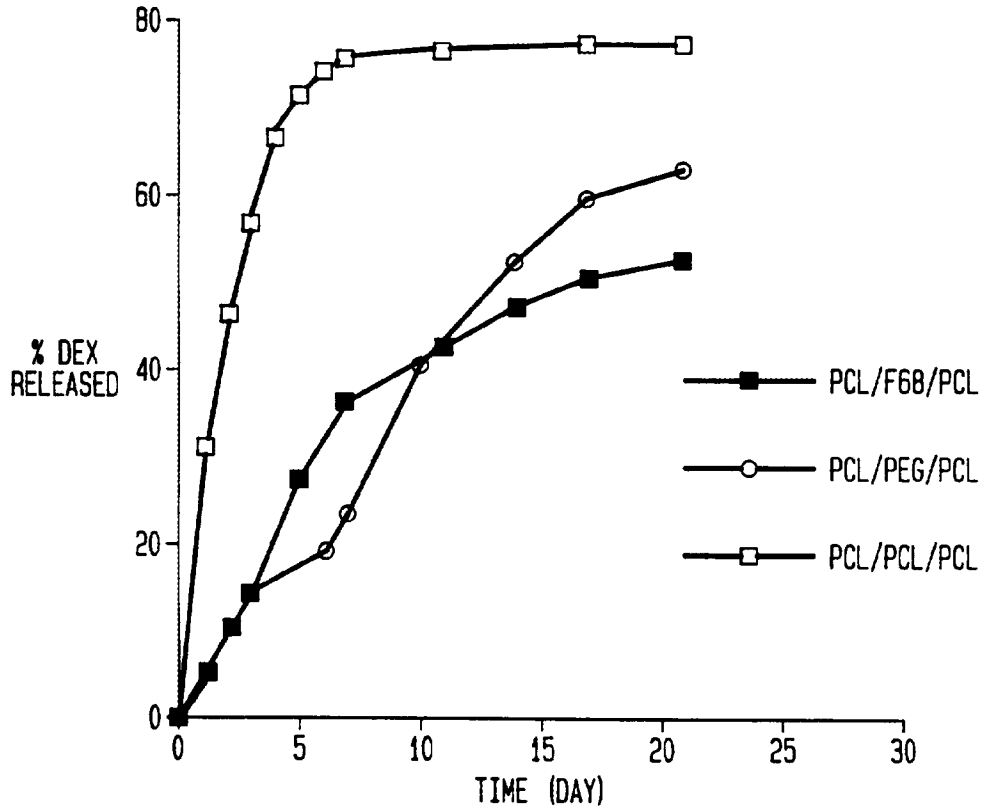

80
60
40
20
0
% DEX RELEASED
0
5
10
15
20
25
30
TIME (DAY)
PCL/F68/PCL
PCL/PEG/PCL
PCL/PCL/PCL

FIG. 19

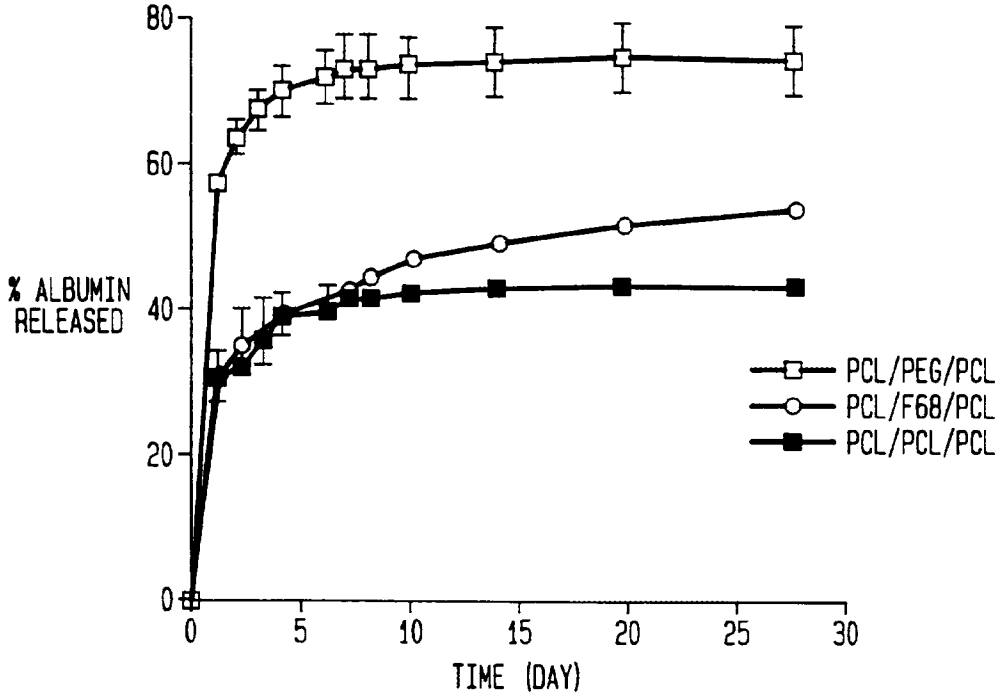

80
60
40
20
0
% ALBUMIN RELEASED
0
5
10
15
20
25
30
TIME (DAY)
PCL/PEG/PCL
PCL/F68/PCL
PCL/PCL/PCL

FIG. 20

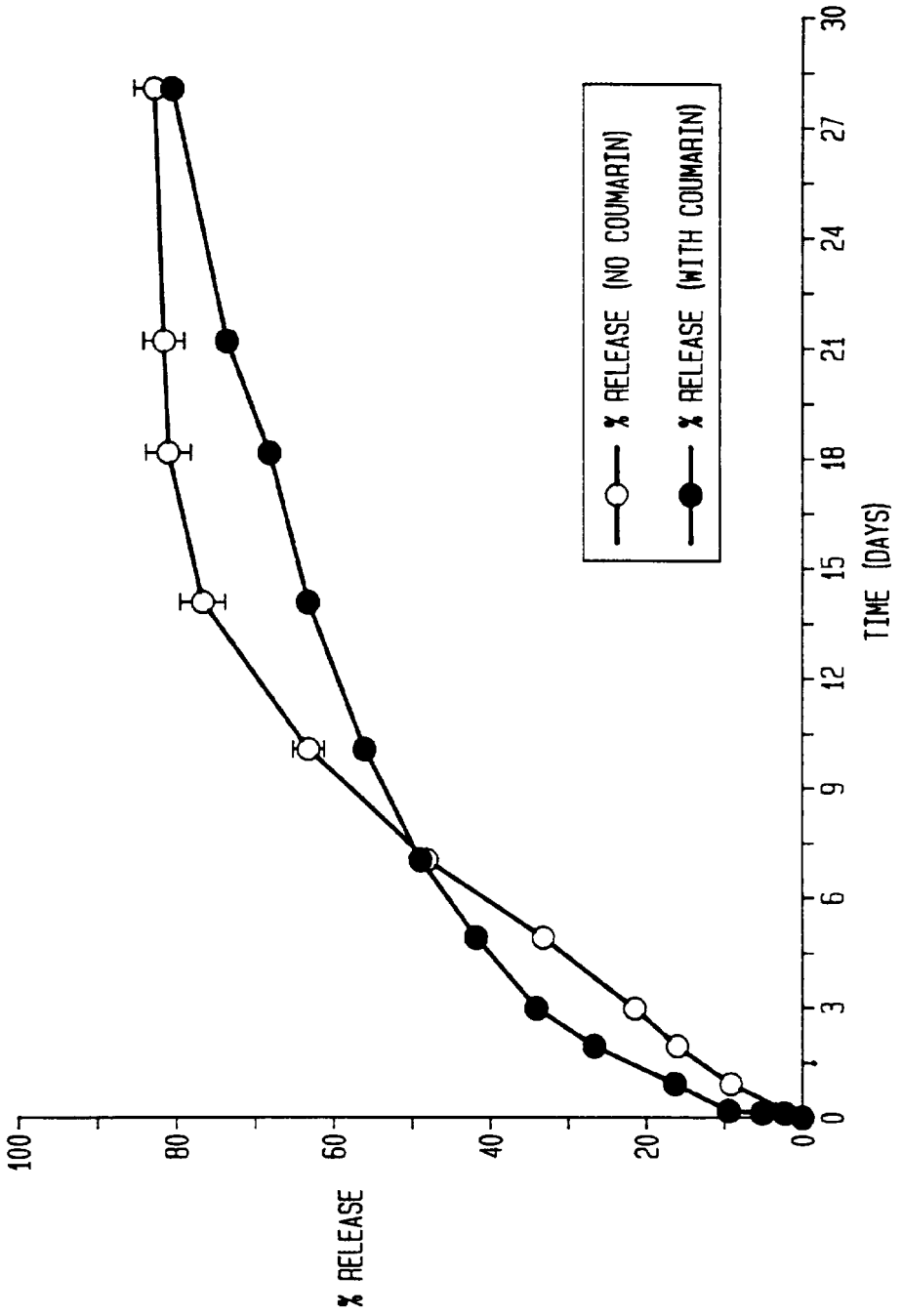

% RELEASE (NO COUMARIN)
% RELEASE (WITH COUMARIN)
% RELEASE
TIME (DAYS)
0
20
40
60
80
100
0
3
6
9
12
15
18
21
24
27
30

FIG. 21

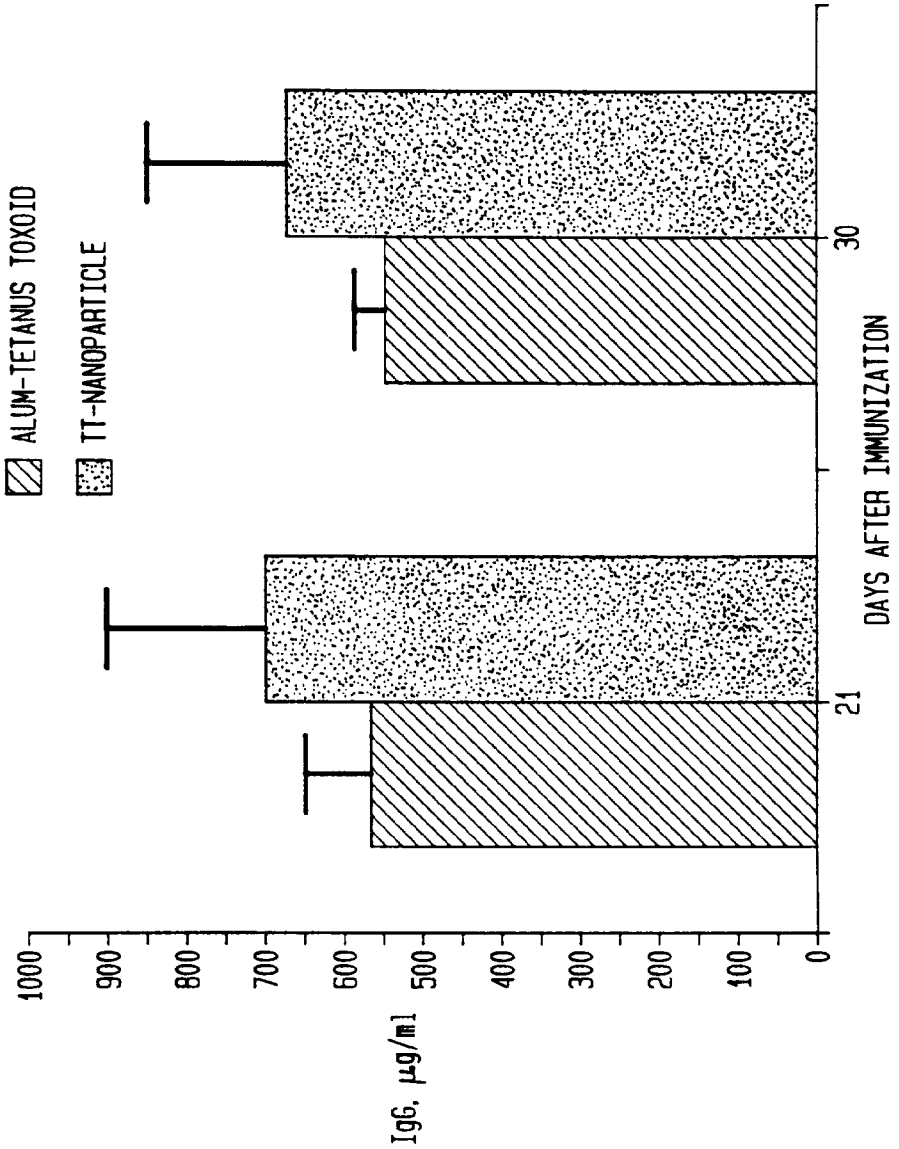
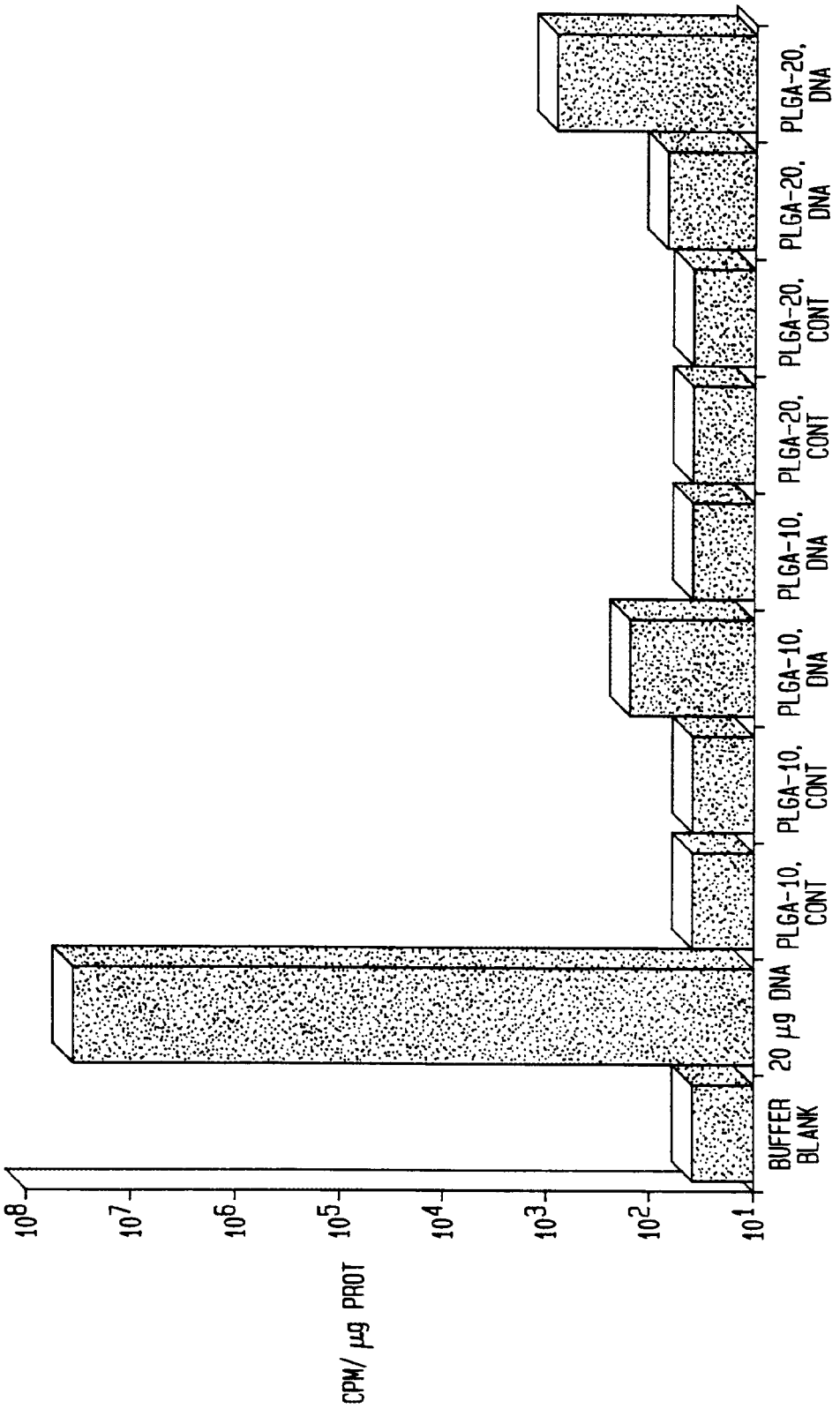

ALUM-TETANUS TOXOID
TT-NANOPARTICLE
1000
900
800
700
600
500
400
300
200
100
0
Igg, µg/ml
21
30
DAYS AFTER IMMUNIZATION

FIG. 22


CPM/ µg PROT
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
BUFFER BLANK
20 µg DNA
PLGA-10, CONT
PLGA-10, CONT
PLGA-10, DNA
PLGA-10, DNA
PLGA-20, CONT
PLGA-20, CONT
PLGA-20, DNA
PLGA-20, DNA